AWARD NUMBER: W81XWH-16-2-0040

TITLE: Polyethylene Glycol Polymers in Low Volume Resuscitation

PRINCIPAL INVESTIGATOR: Martin J Mangino, PhD

CONTRACTING ORGANIZATION: Virginia Commonwealth University
Richmond, VA 23284

REPORT DATE: October 2017

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE
October 2017

2. REPORT TYPE
Annual

3. DATES COVERED
30 Sep 2016 - 29 Sep 2017

4. TITLE AND SUBTITLE

Polyethylene Glycol Polymers in Low Volume Resuscitation

5a. CONTRACT NUMBER
W81XWH-16-2-0040

5b. GRANT NUMBER
BA150349

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Martin Mangino, PhD

E-Mail:

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Virginia Commonwealth
University
1200 E Broad St.
Richmond, VAS 23298

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The effectiveness of polyethylene glycol (PEG) based low volume resuscitation (LVR) solutions is dependent on polymer size, which correlated with their distribution in the microcirculation. Specifically, PEG-20k (MW 20,000) produced optimal resuscitation outcomes compared to smaller or larger polymer sizes. Shocked rats resuscitated with PEG-20k all survived 24 hours (100%) compared to saline volume controls (0%) and had brain function scores comparable to sham controls after recovery from shock. PEG-20k was mainly excreted by the kidneys with a half-life of about 6 hrs. Maximum PEG-20k blood levels (3 mg/ml) were 3 times lower than the lowest dose that produced a mild coagulopathy in ex-vivo blood testing using TEG in volunteers or trauma patients (10 mg/ml). Further coagulation and platelet function studies suggest the mild coagulopathy with higher doses of PEG LVR solutions is due to nonspecific platelet passivation, probably by surface binding, but is clinically moot at currently used concentrations in shock.

15. SUBJECT TERMS

Crystalloid IV fluids, hemorrhagic shock, osmotic effects, oxygen debt repayment, tissue swelling

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT U Unclassified	**b. ABSTRACT** U Unclassified	**c. THIS PAGE** U Unclassified	UU Unclassified		**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Page

1. **INTRODUCTION:** Earlier work from our lab has described a new mechanism of tissue reperfusion injury in shock that is similar to what we had described many years earlier for the preservation of organs for transplantation. Specifically, the metabolic cell swelling of cells that occurs when ATP levels are exhausted during organ ischemia during shock are much more important than previously thought. As energy dependent cell volume control mechanisms are lost during shock, sodium, chloride, and water move into the cell and tissues, which causes metabolic cell swelling. This secondarily compress capillary networks perfusing the tissues and causes further ischemia. More importantly, the compressed microcirculation leads to poor and incomplete resuscitation, which aggravates the problem further. To fix this (and test the hypothesis), we administered cell impermeant molecules to shocked animals to reduce cell swelling by osmotically holding water outside of the cell (because these molecules are impermeant to the cell membrane). This decompresses the capillaries and allows tissue perfusion under low flow conditions and improves outcomes. One complex cell impermeant used was a polymer of polyethylene glycol (PEG-20k), which produced logarithmically better results than any other solution, including standard clinical solutions and our standard impermeant solutions. Our objectives for this project were to 1. Identify probable mechanisms of action of PEG-20k in rodent shock models to support our current osmotic hypothesis and 2. To translate the effects of PEG-20k based low volume resuscitation in a pre-clinical pig model with survival outcomes. A final objective was 3. To test the effects of the PEG-20k based LVR solutions on coagulation and platelet function because prior experiences with polymers in shock resuscitation (hydroxyethyl starch, Hextend) indicate coagulopathies that limit their usefulness in shock and trauma settings. In this year's annual report, we describe mechanistic and survival studies in our proven rodent model of severe hemorrhagic shock with low volume resuscitation (LVR) using PEG-20k based solutions and a comprehensive analysis of the effects of PEG-20k solutions on ex-vivo tests of coagulation and platelet function in volunteer and trauma patient blood. Some of the pharmacological observations in the rodent shock model are used to place the ex-vivo coagulation results into clinical context for upcoming studies in patients.

2. **KEYWORDS:** Crystalloid IV fluids, hemorrhagic shock, osmotic effects, oxygen debt repayment, tissue swelling

3. **ACCOMPLISHMENTS:**

What were the major goals of the project? The major goals of the project were:

I. <u>To identify mechanisms of action of PEG-20k in low volume resuscitation (years 1 and 2)</u>

a.) Determining the effects of PEG polymer size on LVR outcomes to support the hypothesis that only specific sizes of PEG polymers work because they define the unique osmotic reflection coefficient needed to produce the double osmotic gradient in the microcirculation.

b.) Reconstitute the PEG-20k resuscitation effect by using both an oncotic agent with a cell impermeant to validate the double osmotic gradient hypothesis

c.) Determine water movement into tissues after shock using MRI to directly test the cell swelling hypothesis of PEG-20k actions

d.) Determine the effects of PEG-20k in an uncontrolled hemorrhagic shock model to see how the solution performs in uncontrolled bleeding settings

e.) Determine if PEG-20k has effects on TBI which is often known to co-occur in the field with polytrauma and hemorrhagic shock. This tests if the material can reduce brain swelling like it does in other tissues.

f.) Determine if the high arterial pressures created with PEG-20k resuscitation can be titrated and controlled with dosing to control blood loss. This is less of a concern now that the arterial pressures attained in the pig model is much lower than the rat after resuscitation (60 mm Hg Vs. 100 mm Hg), thus reducing the fears of a "pop the clot" effect.

II. <u>To translate effects of PEG-20k to a pig model of controlled and uncontrolled hemorrhagic shock (Years 2 and 3).</u>

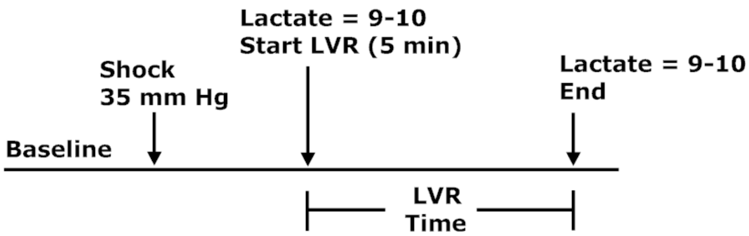
III. <u>To assess PEG-20k based LVR solutions on coagulation and platelet function in blood from volunteers and trauma patients (year 3)</u>

What was accomplished under these goals? Seven projects are described that highlight our accomplishments over the last year.

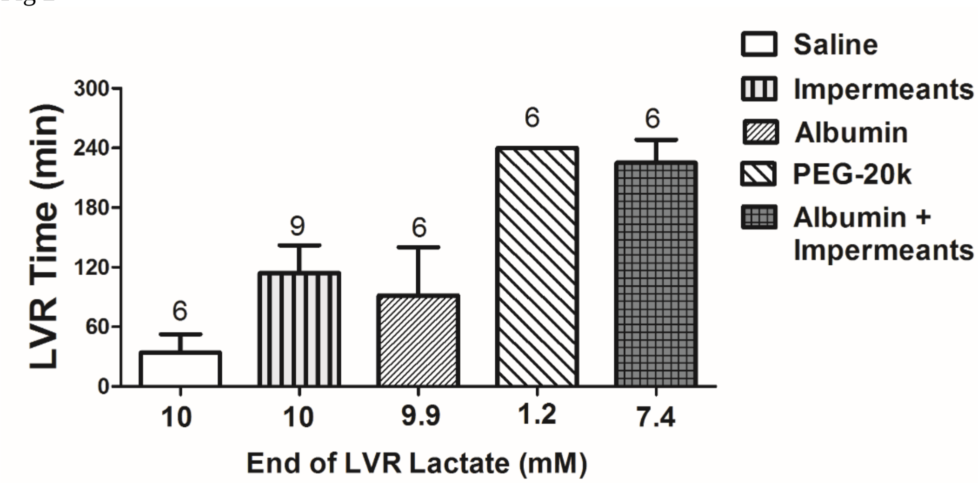
I. Effects of adding impermeants with colloids on LVR times in shocked rats, relative to PEG-20k. Five groups of rats were shocked, given an LVR solution (volume equal to 10% of the calculated blood volume), and the LVR

time determined. LVR time is determined as the time from the start of LVR, which is triggered by lactate between 9-10 mM, until the time when lactate again reached the 9-10 mM limits after LVR. This is described visually in Fig 1.

Figure 1



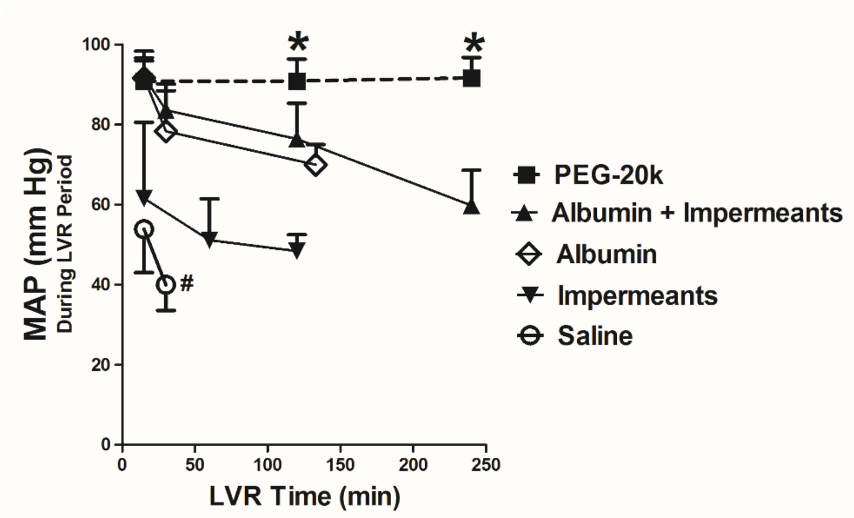
The groups tested included animals resuscitated with LVR solutions containing saline (negative control group), PEG-20k (positive control), and test solutions consisting of an impermeant alone (gluconate), a colloid alone (albumin), and a combination of an impermeant and colloid (gluconate with albumin). The LVR times are shown in Fig 2



Low volume resuscitation with an impermeant increased LVR time (tolerance to the low volume state) 4 fold from 30 to 120 minutes. This was about the same response seen with the colloid albumin. These responses fall far short of the 8 fold increase in LVR time observed with PEG-20k. However, combining both albumin and gluconate increased LVR times equivalent to what was observed with PEG-20k, suggesting that PEG-20k may work, in part, by establishing two osmotic gradients in the microcirculation similar to the two gradients established by the combined solution group in this study. These results support the hypothesis but do not exclude other mechanisms of action for PEG-20k. Furthermore, the true LVR responses between the combined group (LVR solution with both gluconate and albumin) and the PEG-20k group may have been significantly different had we not stopped the LVR period at 240 minutes because the end lactates were significantly different between those groups. Specifically, the lactate in the combined LVR solution group was close to the 9 mM limit while the lactate in the PEG-20k LVR group was far from the limit and continuing to fall towards baseline. This suggests that the true LVR times in the PEG-20k group would be much higher than the true LVR time in the gluconate/albumin group.

II. Effects of adding impermeants with colloids on arterial blood pressure in shocked rats, relative to PEG-20k. Similar to the LVR response, we observed improvements in mean arterial blood pressures when both impermeant and colloid solutions were combined compared to using them separately. The response was not as good as seen with PEG-20k however. The results are shown in Fig 3.

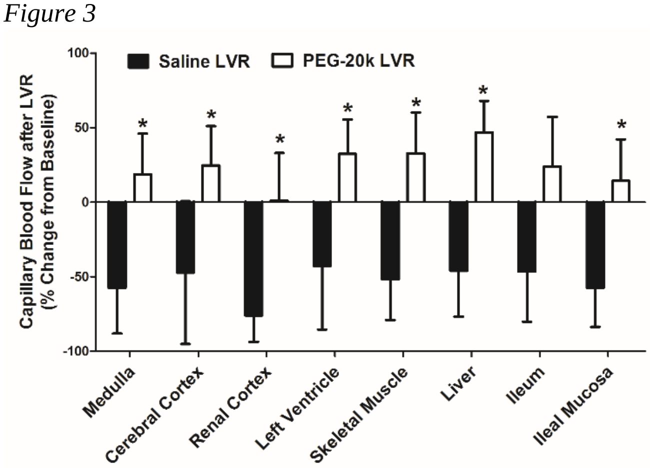
Figure 2



These results support the hypothesis that impermeants draw water out of cells and into vascular spaces, which would predict also lead to an increase in MAP during the LVR period. This drives blood flow in the tissues for oxygen transfer in the low volume state and increases LVR times.

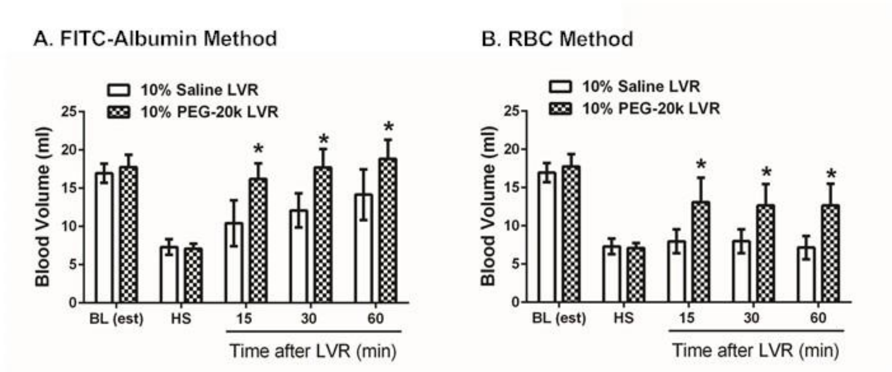
III. **Effects of PEG-20k LVR on capillary blood flow in shocked rats, relative to a saline volume control.** To further support the hypothesis that PEG-20k increases LVR times by moving isotonic water from the extravascular compartment into the vascular space, we measured direct capillary blood flow changes in the LVR period with PEG-20k resuscitation. Capillary blood flow was measured in the LVR period after either saline or PEG-20k resuscitation using colored microspheres. Regional capillary flows are shown in Fig 4 for both groups in many important vascular beds.

Figure 3



Capillary blood flow was significantly higher during LVR in all vascular beds when PEG-20k is added to the LVR solution, relative to the saline vehicle volume control. Again, this further supports our mechanistic hypothesis of an osmotically driven refilling of tissue capillaries that transfers oxygen in the tissues during the low volume state and drive down lactates to increase observed LVR times. In essence, PEG-20k LVR prevents the shock-induced drop in capillary blood flow in important vascular beds. Presumably by increasing the intravascular volume. To address this further, we measured directly the volume in the intravascular space during shock and LVR in these two groups by using two different indicator dilution techniques involving FITC-labeled albumin dilution and red blood cell (hemoglobin) dilution. Figure 5 shows these results.

Figure 4



PEG-20k containing LVR solutions significantly expanded the intravascular volume during shock when the saline vehicle did not. This directly supports the hypothesis on the mechanisms of how these unique polymers work in our shock model.

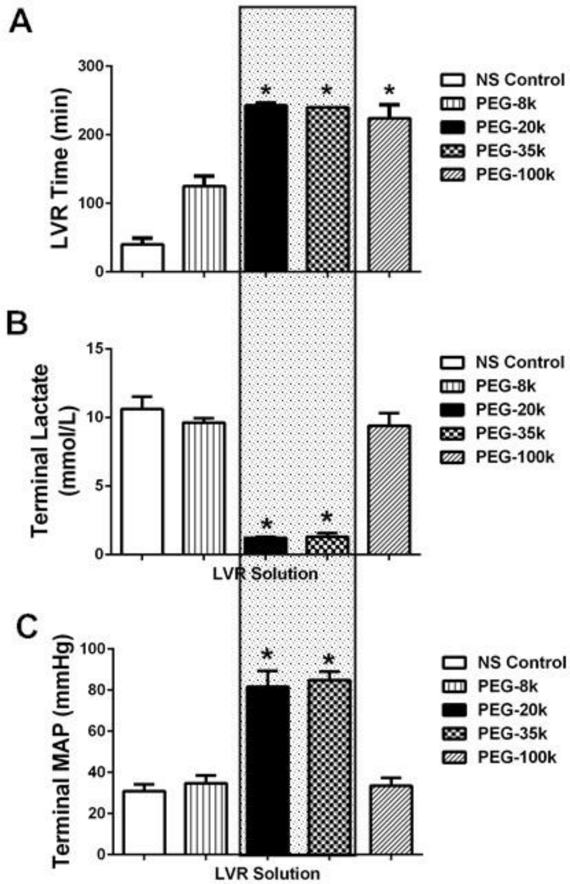
IV. PEG Polymer Size and effects on low volume resuscitation (LVR) from shock.
We hypothesized that PEG-20k is effective because eit has a unique molecular size and radius that allows it to partially but unequally partition in both the capillary and interstitial space while remaining impermeant to the intracellular space. Conversely, it this is true, the larger and smaller size polymers should be less effective. We used (4) sizes of PEG polymers in our standard rodent shock and low volume resuscitation model in acute experiments using low volume resuscitation (LVR) times as the primary outcome. The PEG polymer sizes and groups of rats included;

- Normal Saline (NS) volume control (10% of estimated blood volume) used for LVR
- PEG-8k (10% estimated blood volume) used for LVR
- PEG-20k (10% estimated blood volume) used for LVR
- PEG-40k (10% estimated blood volume) used for LVR
- PEG-100k (10% estimated blood volume) used for LVR

All solutions were used at a 10% concentration of the polymer in saline and administered in a volume of 10% of the estimated blood volume when given for resuscitation. This is equivalent to 500-ml volume for resuscitation of an adult male patient. The protocol for the shock and resuscitation of the rats is shown diagrammatically in Figure 1.

After general anesthesia induction, rats were surgically implanted with vascular catheters and bled through the arterial line until blood pressure reached 35 mm Hg. Pressure was held at this level to increase the oxygen debt, as indexed by the rising plasma lactate level. Once the plasma lactate target of 9-10 mM was reached, a low volume resuscitation crystalloid was given I.V. at 10% of the estimated blood volume over 10 minutes. The blood volume removed to achieve this debt level was typically about 55-60% of the total blood volume, which is a lethal hemorrhage for the volume control (NS) and many other crystalloids such as 10% albumin solution, Hypertonic saline solution, or 6% Hextend solution. The LVR times of each of the groups in this experiment are shown in Figure 6. The higher the LVR time, the more tolerant the rat to the same level of shock.

Fig 6



The LVR time (panel A) is an index of the tolerance of the rats to the low volume state. The more metabolically tolerant they are, the longer it takes them to re-build oxygen debt after a low volume crystalloid resuscitation. The time when they rebuild debt to a lactate of 9 mM again is the time that they require definitive resuscitation to prevent death. Thus, all PEG containing LVR crystalloid solutions significantly prolonged this tolerance period, relative to the volume control with normal saline. A "sweet spot" in PEG polymer molecular weight size was reached between 20-40 kDa where maximum LVR times were seen and where polymers above or below that range were not as protective. This is further exemplified in the data in Figure 6 by comparing the terminal lactates (panel B) and the end systolic blood pressures in the polymer size groups (panel C). Again, PEG-20 to 40k had the lowest end lactate levels and the highest end systolic blood pressures compared to lower polymer sizes (PEG-8k) and higher weights (PEG-100k). It should also be noted that the LVR times for the 20 and 40k PEG polymers were arbitrarily cut off at 240 minutes so their real LVR times were likely much higher, especially since their lactates after 240 minutes were at baseline values (1.2 mM), which are far below the values needed to trigger the end of the LVR time (9-10 mM). Therefore, while all PEG polymer sizes tested were much better than saline, the dramatic effects seen previously are in fact dependent on a strict molecular weight range, which we hypothesize establishes the mechanism of action of this new impermeant class polymer in shock and low volume resuscitation. More specifically, we hypothesize that this strict molecular weight range allows the PEG polymers to have, at equilibrium, partial and unequal excursions of the molecules outside of the capillary space while maintaining many molecules in the capillary space too. This would be defined by the osmotic reflection coefficient that was next characterized in healthy anesthetized rats.

To precisely test this hypothesis and measure the distribution of polymer molecules outside the capillary in the interstitial space versus inside the capillary space, we measured the osmotic reflection coefficients for each of these polymer sizes. A reflection coefficient of 0 means the molecules are not reflected back into the capillary at all and are free to equilibrate evenly between the capillary space and the interstitial space (Figure 7). On the other hand, a reflection coefficient 1 means all of the molecules are reflected back into the capillary and are blocked from diffusing out or blocked from being "dragged" out of the capillary by solvent (water) convection. The osmotic reflection coefficient is calculated by: $\sigma_d = 1-$ [L/P], where [L/P] is the lymph to plasma concentration of the test compound at high lymph flow rates (volume loading) conditions. The test polymers used tracer polymers containing FITC so activity in lymph and plasma is monitored by detecting fluorescence using an excitation-emission spectrofluorometer. The ramifications of having a unique solute that is both impermeant to cells AND unequally partitioned into both the interstitial and capillary spaces, i.e., has a reflection coefficient greater than 0 but less than 1 is that two distinct osmotic gradients are established outside of the cell to drive the transfer of free water from the metabolically swollen cell into the capillary space where it belongs. This water transfer then decompresses capillary beds by decreasing the transmural pressure on them that tends to reduce their effective radius and limit flow while increasing capillary volume and pressure that supply the driving force for capillary flow. Figure 8 shows the results of the osmotic reflection coefficient studies with polymer sizes of PEG-8k, PEG-20k, PEG-40k, and one experiment with PEG-100k.

Figure 7

of 1of
thethe
pores
where
ratio
were

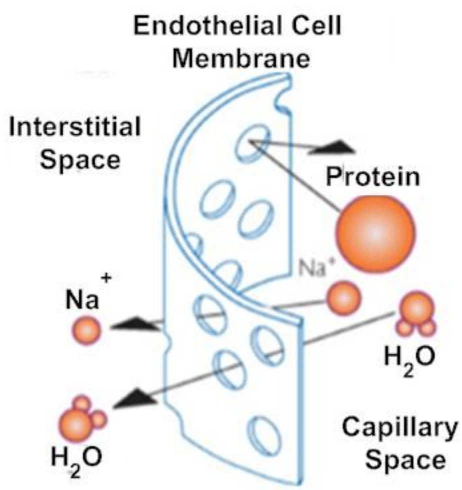
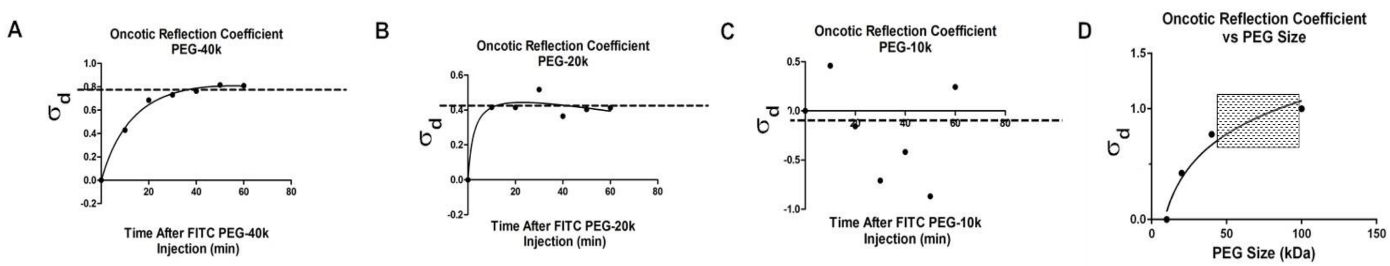


Figure 8

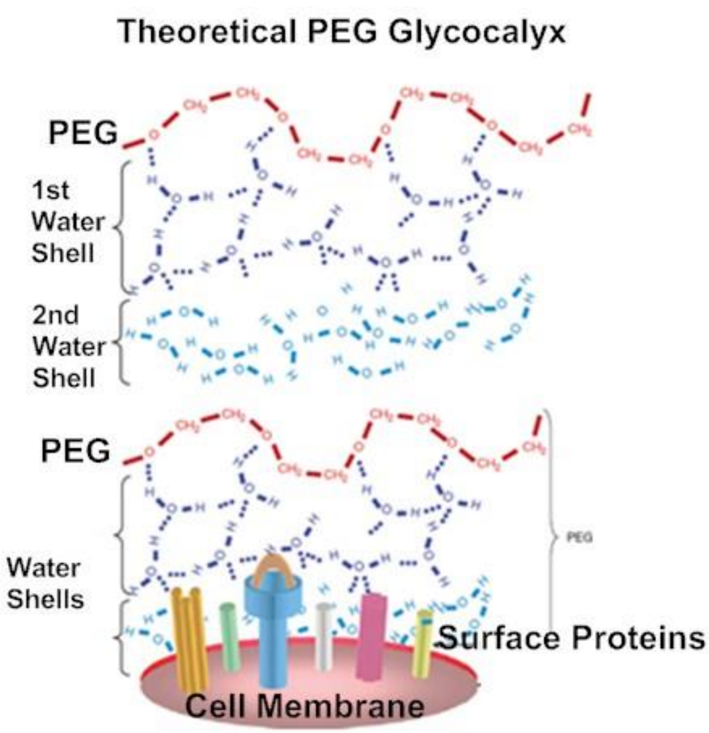


In Figure 8, the osmotic reflection coefficients are shown over time after the injection of the fluorescently labelled tracer PEG polymer. It generally takes 10-20 minutes to achieve a steady state as seen by the flat lines, which represent the true osmotic reflection coefficients. No raw data are shown for PEG-100k because the commercial material was found to be contaminated with smaller labeled polymers besides 100k so we had to purify the material by size exclusion filtration before use. This is why the curve in Panel D shows a shaded box from PEG 40k to PEG-100k because we have very limited data and the results between those points is less certain. In panel A, the partitioning of PEG-40k is shown to be about 0.8, which means that for every 4 molecules that stay in the capillary space, 1 moves into the interstitium (and 0 go into the cell). Panel B shows the same coefficient data for PEG-20k and recapitulates the osmotic reflection coefficient of 0.4, which means that for every 5 molecules of PEG-20k that stay in the capillary space, 2 wander out into the interstitial space (and 0 enter the cell). Figure 4C show the

partitioning for PEG-8k with a coefficient of essentially 0 (negative numbers are actually unachievable and represent sampling error). This means that for any number of PEG-8k molecules that stay in the capillary, the same number migrate into the interstitial space (and 0 enter the cell) because the material is freely permeable to the capillary membrane. Finally, for PEG-100k, our currently limited data suggest a coefficient around 0.9-1.0, which means that almost all of the PEG-100k molecules are confined to the capillary space with only limited numbers (<10%) being partitioned into the interstitial space (and 0 moving into the cell). This material behaves as a classic colloid. These coefficient data, together with the shock outcomes data (Figure 6), indicate that the most effective PEG polymers are between 20-40 kDa and possess a reflection coefficient between 0.4-0.7. This by definition describes the establishment of multiple osmotic gradients by the most effective PEG polymer sizes and supports our hypothesis regarding conditions for effective energy independent unidirectional osmotic water transfer from cell to capillary.

Because the polymer sizes used in these studies are large, the actual numbers of molecules and osmotically active particles partitioning into these spaces is relatively small. So, the osmotic gradients established are relatively small (mOsM concentrations in the single digits). However, the relative attraction for water molecules by PEG polymers is large as they are known to form multiple water "shells" around the PEG polymer backbone (Figure 9). This likely explains why relatively small osmotic gradients of PEG have such huge water transfer properties.

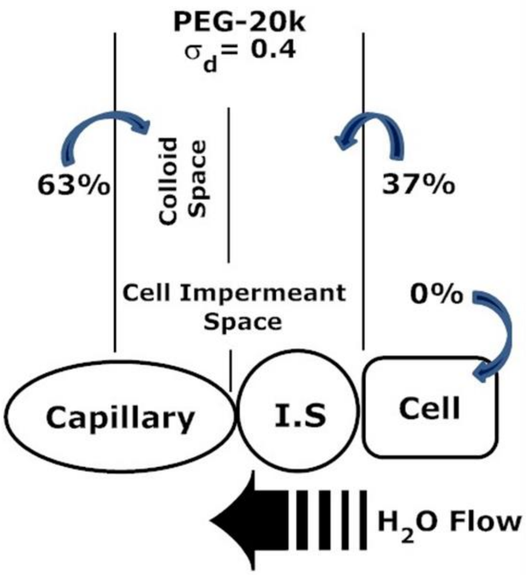
Figure 9

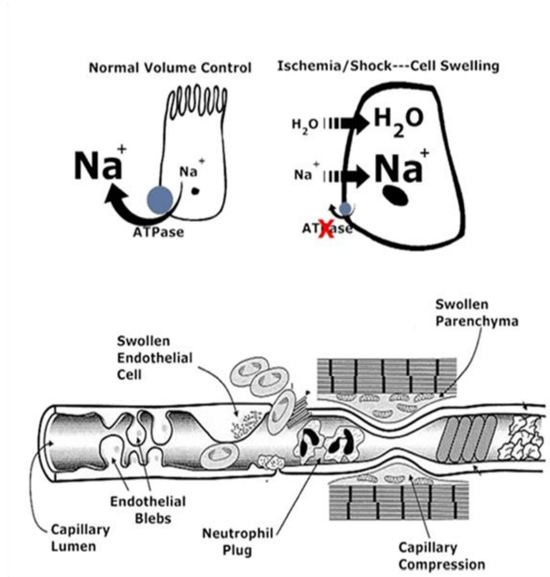


As is also shown in Figure 9, the PEG polymers are capable of binding to and possibly repairing the endothelial cell glycocalyx. Since the glycocalyx is likely injured in severe shock and low volume resuscitation, we will in the future explore whether some biological effects of PEG-20k based LVR solutions are partly attributable to rebuilding and rehydrating the damaged glycocalyx.

At this point, we have identified a likely hypothesis to explain how PEG-20k works in LVR. Based on our presented data, we believe that the unique osmotic reflection coefficient for PEG-20k establishes 3 osmotic gradients in the microcirculation that efficiently and non-energetically moves metabolic water out of the cells and into the capillary spaces. The result is to prevent cell swelling injury, decompress the microcirculation, reload the capillaries, and dramatically improve capillary flow and oxygen transfer under very low volume conditions. **Figure 10** shows this diagrammatically.

Figure 10





V. Effects of PEG-20k LVR solutions on shock survival and neuro-cerebral function

We have clearly demonstrated in acute experiments in the rodent and pig model how PEG-20k LVR solutions increase the tolerance to the low volume state 20 fold over saline volume controls, based on the low volume resuscitation time. In the next study we determined if there was a translational survival benefit to being resuscitated with PEG-20k LVR as indexed by next day survival in rats treated with PEG-20k compared with the saline controls. The table shows the survival increase from 0% (saline) to 100% (PEG-20k), neuro-deficit scores that remain normal for PEG-20k treated rats (0 = normal, 500 = brain dead), urine production, day 2 mean arterial pressures (MAP), and day 2 lactates. The rats experiencing severe lethal hemorrhagic shock are not different compared to sham operated rats using these outcomes. But they are vastly improved over the shocked rats receiving saline (0% survival), In addition to surviving the lethal shock, PEG-20k resuscitated rats had normal brain function (NDS).

TABLE 1: Rodent 24 hour survival data after lethal hemorrhagic shock in PEG-20k and normal saline (NS) resuscitated groups. UOP=Urine output, MAP=mean arterial pressure.

Group (n)	Survival (%)	NDS	UOP (cc/kg/hr)	Day 2 MAP (mmHg)	Lactate (mmol/L)
PEG 20k (8)	100	64	2.8	86	2.7
Sham (1)	100	40	5.11	109	2.8
NS (5)	0	N/A	N/A	N/A	N/A

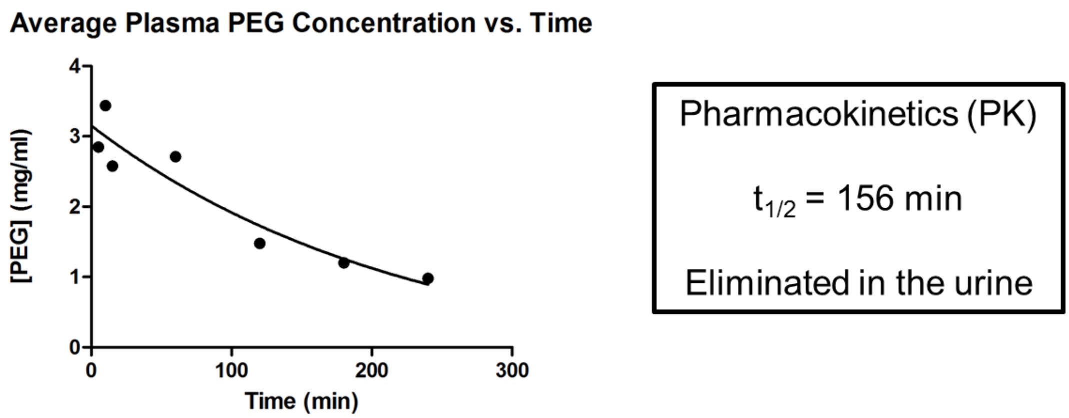
NDS - Neurologic Deficit Score: 0 – normal, 500 – brain dead. Values are given as means.

VI. Plasma elimination of PEG-20k after resuscitation from lethal hemorrhagic shock

The elimination and blood levels of PEG-20k were monitored in these animals over 24 hours to determine the half-life of elimination and to determine the exact blood levels immediately after resuscitation. These initial blood levels represent peak levels and are the maximum levels use to compare our ex-vivo platelet function and coagulation study results that were conducted in parallel studies. PEG-20k is administered as a theoretical 10% dilution of the calculated circulating blood volume. Therefore, administration of a solution with a concentration of 100 mg/ml of PEG-20k should dilute to 10% immediately after

administration and achieve theoretical levels of 10 mg/ml. Therefore, we tested PEG-20k in whole blood at 10 mg/ml for coagulation and platelet function using Thromboelastography (TEG) and other platelet and coagulation factor specific assays (described in the next section and in two appended manuscripts). The results of the blood level study are shown in figure 11 and are derived using a FITC-labelled PEG-20k probe to detect blood levels over time in shocked rats.

Figure 11



Important features to note are the initial blood levels (3 mg/ml) and the half-life of over 2 hours. The initial blood levels are **3 times lower** than the blood concentrations used in the coagulation studies. This will be important to remember when those results are presented. Also, the half-life is rather short for a single administration agent so concerns about prolonged exposure and toxicity are alieved.

VII. Coagulation and Platelet function effects of PEG-20k LVR solutions in ex-vivo human blood

The use of polymers in crystalloid resuscitation solutions is not new and previous solutions using plant dextrans (Rheomacrodex) or hydroxyethyl starch (Hextend) as volume expanders have had disappointing results. In fact, the side effects of these polymers on coagulation and platelet function, the immune system, and the kidneys has led to Rheomacrodex being removed from the market by FDA and Hextend being essentially shunned for use by trauma and Emergency Medicine professionals because of the toxicity in already critical and sick patients. Therefore, although the resuscitation outcomes of PEG-20k are logarithmically superior to these solutions, one still worries that similar side effects occur. Therefore, we explored in detail the effects of PEG-20k low volume resuscitation solutions on coagulation and platelet function in ex-vivo blood obtained from both healthy volunteers and from very sick trauma patients early after entering our trauma system at VCU. These data are extensive and they have been recently submitted as two companion manuscripts to the journal SHOCK for consideration for publication. Both companion manuscripts are attached in the appendix and won't be fully described here in this section of the report.

As a brief summary, whole citrated blood was drawn fresh from a group of 46 healthy volunteers that were free of any medications, had no know blood clotting disorders, and were healthy at the time of donation. Another population of 22 trauma patients were also studied. These patients had just arrived to the Emergency Medicine Department of VCU Medical Center and received no IV fluids or a maximum of <500 ml of either LR or saline IV. These patients were drawn in the first hour of arrival to the ED. They had a plasma lactate of >4.6 mM, a systolic blood pressure of 95 mmHg or less, and an Index Severity Score (ISS) of >24 (determined retrospectively). All blood was analyzed by Thromboelastography, light transmission aggregometry, FACS, Coagulation assays, and a variety of other assays to dissect out whether any coagulation or platelet function effects could be identified and what mechanisms could account for the defects. Blood was first diluted 1:9 with various IV crystalloids before analysis. These included saline or LR as a volume control (10%), Hextend (10% dilution of a commercial 6% solution), PEG-20k (10% solution at a 10% dilution), and PEG-20k (7.5%

solution at a 10% dilution). The results indicate that PEG-20k used at a 10% dilution, which is based on the theoretical dosing of shock patients at a volume equal to 10% of the estimated circulating blood volume, produces a mild hypocoagulative state on TEG. Further evidence indicates the response is a nonspecific platelet passivation or thrombasthenia, possibly to the masking of IIb/IIIa receptors. No effects on coagulation are seen. However, standard silica used to activate the intrinsic coagulation pathway and measure aPTT INR are consumed or blocked by the presence of PEG-20k in the samples. This prevents activation of intrinsic coagulation and artifactually prolongs the aPTT times and individual factors in that pathway like fVIII. This provides completely erroneous information about intrinsic coagulation and could lead to panic and incorrect treatment. Therefore, when these solutions go into clinical trials and practice, it will be important for an FDA notice to be issued to clinical laboratories of this so they know they have to use **non-silica based activators for aPTT testing**. We recommend Kaolin activation, which gives normal aPTT results with blood treated with PEG-20k based solutions. In further studies, we saw the same effects of 10% PEG-20k on blood from normal volunteers as we did trauma patients. Specifically, at a 10% dilution, a slightly hypocoagulative state was seen in both groups, which was characterized by a slight but significant reduction in MA on TEG, which is mostly a platelet effect. However, this response was dose dependent and completely disappeared when the PEG-20k concentration was reduced to 7.5% from 10%.

At a 10% dilution of a 10% concentration of PEG-20k, the theoretical concentration of PEG-20k in the circulating blood should be about 10 mg/ml (10% dilution of 100 mg/ml solution). However, when we empirically measure the peak concentrations of PEG-20k in the circulation of shocked animals immediately following resuscitation, the [PEG-20k] was consistently determined to be about 3 mg/ml. Since this level is 3 fold lower than the 10 mg/ml that produces the hypocoagulative state on TEG, the chances for this occurring in trauma patients is very small. In fact, preliminary TEG data from 4 shocked pigs receiving a 10% dilution of PEG-20k (containing a fluorescent PEG-20k probe) indicate completely normal coagulation and platelet function tests (TEG, data not shown). Therefore, the slight but significant platelet passivation effects of higher doses of PEG-20k are not clinically relevant and should not be a clinical concern. The 3 fold difference between maximum blood levels that we see in shock and the minimum blood levels necessary to see the coagulation effect provide a huge safety margin (therapeutic index) for PEG-20k based LVR solutions when used for shock resuscitation.

What opportunities for training and professional development has the project provided? The laboratory actively trains surgical residents, non-surgical residents, post-doctoral fellows, graduate students and junior faculty in the school of medicine. These people that participated in the conduct of the studies were given extensive opportunities to learn how to technically conduct shock and laboratory studies, analyze data, design experiments, and present and publish results. In this annual report period, Dr Nina Wickramaratne and Dr. Loren Liebrecht participated as resident surgeon and post-doctoral trainee, respectively. Dr. Wickramaratne will go back on service this July and Dr Liebrecht started her residency training in Internal Medicine last July. This July, new trainees will move into these positions to continue the work on the project, under the direct supervision of Dr. Mangino and his staff.

How were the results disseminated to communities of interest?
The results of the last year have been widely disseminated at local VCU seminars and training competitions, at national and international shock and trauma societies, and at the American College of Surgeons meetings. These data are also disseminated by publication in the scientific literature.

What do you plan to do during the next reporting period to accomplish the goals?
Similar studies are planned to continue progress on the goals of the project. Shock studies in the rodent and pig model will be used to assess survival after LVR with PEG-20k solutions. Additional studies on uncontrolled shock will begin in both rat and pig studies. MRI work in the rat model will start to measure local tissue water transfer and osmotic reflection coefficients of PEG polymers of various sizes will be

determined in the brain circulation to determine if PEG polymers can be used and customized in size to work in neurotrauma to prevent TBI.

4. **IMPACT:**

The impact of this project and these results are huge in trauma resuscitation, especially in pre-hospital setting for military and civilian shock. The finding of the safety of the PEG-20k based LVR solutions at effective concentrations has a great impact on moving the commercialization and FDA approval forward in plans for deployment. Since these studies are IND-enabling and because we are designing the experiments with FDA approval in mind, we feel we have a significant leg up in this area. Our plans are for a pre-IND meeting with FDA this winter. A phase-1 dose escalation trial has already been designed for use in normal blood donors. The impact of these data to date on our IND application are very significant.

What was the impact on the development of the principal discipline(s) of the project?

We believe that these data reorient our thinking about the major mechanisms of tissue reperfusion injury. The old ideas of free radical injury and inflammation should give rise to the major importance of metabolic cell and tissue swelling and the "no reflow" phenomenon because of the huge biological effect that we repeatedly see with specifically sized PEG polymers in shock and local I/R injury models. This reorientation in thinking about the major mechanism of reperfusion injury is strengthened by our "locking down" of the mechanisms of how this material works in our shock models. Our experiments to date have incorporated mechanistic studies that clearly suggest the effects are due to the non-energy dependent movement of intracellular water out of tissues and into capillary spaces in order to decompress the local microcirculation and allow for extremely efficient oxygen transfer in the microcirculation under very low volume states as seen in severe hypovolemic shock (figure 10). This reorientation of thinking also helps us design smarter solutions in the future and in customized tissue beds like CNS to mitigate reperfusion injury in spinal cord injury and TBI

What was the impact on other disciplines?

As discussed above, the implications for reperfusion injury in any other aspect of medicine are applicable to this work since they may all share similar mechanisms of tissue reperfusion injury. Clearly, these developments will be used to treat pre-hospital shock but a myriad of other conditions that all involve derangements in volume control secondary to tissue ischemia. Such applications being considered by VCU include abdominal and extremity compartment syndrome, neuro-trauma such as spinal cord injury and TBI, volume control in cardiopulmonary bypass and ECMO, CPR, prevention of critical illness in the ICU, ED resuscitation, septic shock, burn resuscitation, pre-hospital trauma resuscitation in civilian and military transports, peripheral vascular disease and chronic heart failure, and space medicine.

What as the impact on technology transfer?

Technology transfer is a very important aspect of this work. Every study that reinforces the clinical importance, biological mechanisms, and safety profiles of the new LVR solutions and platform, improves tech transfer for deployment.

What was the impact on society beyond science and technology?

Whenever healthcare can be impacted to this degree, society benefits from a more productive, economical, and fruitful life for its members.

5. **CHANGES/PROBLEMS:**

There are no problems in the project. In the original study proposed to test the dose response of PEG-20k to prevent over pressure during resuscitation is now moot since we discovered that over pressure with PEG-20k only happens in the rodent model. Where the mean arterial pressure would rise to 100

mmHg after resuscitation in the rat, which may cause more bleeding in uncontrolled shock, mean arterial pressures only rose to about 60-65 mmHg in the clinically relevant porcine shock model. Since the pig represents a more real picture for patients, we feel that 60 mmHg is the perfect pressure that allows good local perfusion but limits further bleeding or risk of "pop the clot" effects. Therefore, we feel it is more important to use the saved resources from not conducting those studies to engage in more IND enabling studies like longer term survival and acute toxicity studies, or whatever FDA may ask of us following our pre-IND meeting. .

Changes in approach and reasons for change

See above

Actual or anticipated problems or delays and actions or plans to resolve them

None.

Changes that had a significant impact on expenditures

None

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None

Significant changes in use or care of human subjects

None

Significant changes in use or care of vertebrate animals.

None

Significant changes in use of biohazards and/or select agents

None

6. **PRODUCTS:**

None to report, outside of the already technology being developed and commercialized

Publications, conference papers, and presentations.

Journal publications.

1. Plant V., Parrish D., Lindell S, Limkemann A, Reichstetter H, Ferrada P, Aboutanos M., and **Mangino MJ.** Low volume resuscitation in hemorrhagic shock: Understanding the mechanisms of PEG-20k. J. Pharm. Exp. Ther. 361 (2): 334-340, 2017
2. Liebrecht, L., Newton, J., Wickramaratne, N., Jayaraman, S., Han, J., Aboutanos, M., and **Mangino, M.J**. Thromboelastographic Analysis of Novel Polyethylene Glycol Based Low Volume Resuscitation Solutions. SHOCK. Submitted
3. Liebrecht, L., Newton, J., Martin, E., Brophy, D., Wickramaratne, N., Jayaraman, S., Han, J., Aboutanos, M., and **Mangino, M.J**. Mechanistic Analysis of the Coagulation and Platelet Function Profile of Novel Polyethylene Glycol Based Low Volume Resuscitation Solutions. SHOCK. Submitted

Books or other non-periodical, one-time publications.

1. **Mangino, MJ**, Liebrecht, L, Plant, V., Limkemann, A.
Crystalloid and colloid resuscitation-Hypertonic saline, starches, polymers, gelatins.
In: Hemorrhagic Shock: Recognition, Pathophysiology and Management
Nova Science Publishers, Chapter 8
Jose Pascual and Jeremy Cannon, Editors
2016,

Other publications, conference papers, and presentations.

Presentations at National Meetings:

1. Wickramaratne, N., Microcirculatory Effects of Polyethylene Glycol 20,000 During Resuscitation of Hemorrhagic Shock. American College of Surgeons
October 2017

2. Wickramaratne, N., Microcirculatory Effects of Polyethylene Glycol 20,000 During Resuscitation of Hemorrhagic Shock. Shock Society
June 2017

3. Wickramaratne, N., Microcirculatory Effects of Polyethylene Glycol 20,000 During Resuscitation of Hemorrhagic Shock. Military Health System Research Symposium (MHSRS)
August 2017

4. Liebrecht, L., Ex-vivo coagulation analysis of polyethylene glycol 20,000 (PEG-20k) resuscitation solutions using thromboelastography. Shock Society
October 2017

5. Liebrecht, L., Ex-vivo coagulation analysis of polyethylene glycol 20,000 (PEG-20k) resuscitation solutions using thromboelastography. Military Health System Research Symposium (MHSRS)
August 2017

Website(s) or other Internet site(s)

None

Technologies or techniques.

None

Inventions, patent applications, and/or licenses

None

Other Products

None

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?

No Change

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award).*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

None

8. **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:** *None*
- **QUAD CHARTS:** *N/A*

9. **APPENDICES:** *Attached and includes*

I. Combined PDF of (3) published papers since the beginning of the project

Appendix

1521-0103/361/2/334–340$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

https://doi.org/10.1124/jpet.116.239822
J Pharmacol Exp Ther 361:334–340, May 2017

Low-Volume Resuscitation for Hemorrhagic Shock: Understanding the Mechanism of PEG-20k

Valerie Plant, Dan W. Parrish, Ashley Limkemann, Paula Ferrada, Michel Aboutanos, and Martin J. Mangino

Organ Preservation Laboratory, Department of Surgery, Division of Acute Care Surgical Services (V.P., D.W.P., P.F., M.A., M.J.M.), Department of Physiology and Biophysics (M.J.M.), and Department of Emergency Medicine (M.J.M.), Virginia Commonwealth University, School of Medicine, Richmond, Virginia

Received December 28, 2016; accepted March 6, 2017

ABSTRACT

Hemorrhagic shock leads to cell and tissue swelling and no reflow from compressed capillaries. Cell impermeants, including polyethylene glycol-20,000 (PEG-20k), reverse ischemia-induced cell swelling, extend low-volume resuscitation (LVR) time after shock, and increase tolerance to the low-volume state. The purpose of this study was to explore the mechanisms of action of PEG-20k containing LVR solutions. We hypothesized that PEG-20k acts as both an oncotic agent and an impermeant in the microcirculation, which moves water out of the space and into the capillaries to affect peripheral capillary filling and enhanced perfusion during the low-volume state. Rats were hemorrhaged until arterial lactate reached 9–10 mM/liter. Then, saline-based LVR solutions containing various impermeant materials were administered (10% blood volume). The LVR times for these solutions were determined by measuring the amount of time required for plasma lactate to climb back to 9 to 10 mM after LVR administration (low-volume tolerance). Capillary blood flow was measured by colored microspheres, and blood volume was measured by fluorescein isothiocyanate–labeled albumin dilution. Gluconate (impermeant), albumin (colloid), and PEG-20k (hybrid) increased LVR time over saline by 4-, 3-, and 8-fold, respectively. The combination of impermeant + albumin produced a biologic effect that was similar to PEG-20k alone. Capillary blood flow and plasma volume were decreased after shock with saline LVR but increased with PEG-20k, relative to saline. These data are consistent with the hypothesis that PEG-20k may act by establishing multiple osmotic gradients in the microcirculation to drive cell-to-capillary water transfer during hypovolemic shock.

Introduction

Minimizing the use of crystalloids and using blood products after trauma are now becoming mainstream in civilian trauma centers. Damage-control resuscitation is also emerging as the standard of care for the US Department of Defense, according to the Joint Theater Trauma Systems Clinical Practice Guidelines. When blood products are not available for resuscitation, crystalloid solutions are administered; however, only a fraction of infused crystalloid volume stays in the intravascular space, and the use of low-volume crystalloids has minimal effects on pressure and perfusion (van Lambalgen et al., 1990; Parrish et al., 2015a). The movement of crystalloid fluid from capillary to interstitium is compounded by the increase in capillary permeability from trauma-related inflammation and trauma-induced capillary leak syndrome (TICS) (Stein and Scalea, 2012). Furthermore, crystalloid resuscitation exacerbate TICS, acidosis, hypothermia, and coagulopathy (Duchesne et al., 2010; Stein and Scalea, 2012). Other resuscitation solutions, such as hypertonic saline or starch, have had disappointing results (Riha et al., 2011, 2013), including concerns and risks associated with their use (Cotton et al., 2006; Duchesne et al., 2010). There remains a need for a better crystalloid fluid that can be given at a low volume to resuscitate patients in hemorrhagic shock awaiting definitive treatment, especially for the prehospital setting. This study tested possible mechanisms of such a solution.

The dominant mechanism of injury in hemorrhagic shock is energy failure secondary to a lack of end-organ perfusion and loss of adequate microvascular oxygen transport with subsequent loss of aerobically produced ATP (Chaudry et al., 1974). As cells lose ATP owing to ischemia, the sodium pump shuts off and sodium ions enter the cell and accumulate as they run down their electrochemical gradient. Chloride follows electrogenically, and water enters the cell osmotically. As water enters ischemic cells, they swell and compress nearby vascular structures, which further aggravates ischemia by reducing local microcirculatory flow (Reffelmann and Kloner, 2002; Rezkalla and Kloner, 2002; Kloner, 2011). Swollen vascular endothelial cells and parenchymal cells compress capillaries to cause no-reflow, promote resuscitation injury, and limit oxygen delivery during the low-flow state and after

This work was supported by grants from the Department of Defense (W81XWH-12-1-0599) and (W81XWH-16-2-0040) to Dr. Mangino.

This study was presented, in part, at the 74th Annual Meeting of the American Association for the Surgery of Trauma, September 9–12, 2015, in Las Vegas, Nevada.

https://doi.org/10.1124/jpet.116.239822.

ABBREVIATIONS: FITC, fluorescein isothiocyanate; IL-1β, interleukin-β, LVR, low-volume resuscitation; PEG-20k, polyethylene glycol-20,000k; TICS, trauma-induced capillary leak syndrome.

full resuscitation (Reffelmann and Kloner, 2002). Reversal of cell swelling with cell impermeants has been used successfully in organ preservation for transplantation (Southard and Belzer, 1980) and in shock (Mees et al., 1982; Parrish et al., 2015a,b). These molecules are permeable to the capillary but impermeable to the parenchymal cell attributable to size and charge, thus creating an extracellular osmotic gradient that inhibits water entry into the cell.

Parrish et al. (2015a) have demonstrated reduced ischemia-induced cell swelling, increased tolerance to the low-volume state, and higher survival rates with administration of cell impermeant-based low-volume resuscitation (LVR) solutions in a rodent model of severe hemorrhagic shock. It was reasoned that if this occurs because of the creation of an osmotic gradient for fluid movement during ischemia, then a second gradient created with the addition of an oncotic agent in the resuscitation solution would augment the response. Indeed, when gluconate (a cell impermeant) was combined with polyethylene glycol 20,000 (PEG-20k, a colloid) in a LVR crystalloid solution, a marked potentiation in low-volume tolerance and blood pressure was observed (Parrish et al., 2015a). Surprisingly, when PEG-20k was used alone, it was equally effective as PEG-20k with the impermeant (gluconate). Additional studies demonstrated that PEG-20k, originally believed to be an oncotic agent, has both oncotic and impermeant effects because some of the material escapes the capillary space (impermeant effects) (Parrish et al., 2015b). This rather rare molecular behavior may explain how PEG-20k alone increased the LVR time (tolerance to the low volume state) 8-fold compared with either saline, mixtures of cell impermeants alone or pure oncotic agents alone (albumin). Specifically, this one agent may be doing double duty as both an oncotic and an impermeant molecule to generate a double gradient for fluid movement in the microcirculation. Therefore, we hypothesized that PEG-20k in shock acts via biophysical effects on water movement in the microcirculation through both cell impermeant and oncotic properties. These properties prevent cell swelling during ischemia, reload the capillaries with isotonic fluid from the interstitial space, and decompress the microcirculation, which all leads to increased capillary perfusion and oxygen transfer in the low-volume state. Figure 1 shows the hypothesized biophysical mechanisms of PEG-20k–based LVR solutions in low-flow and shock states. Here we present the results of experiments that support this hypothesis.

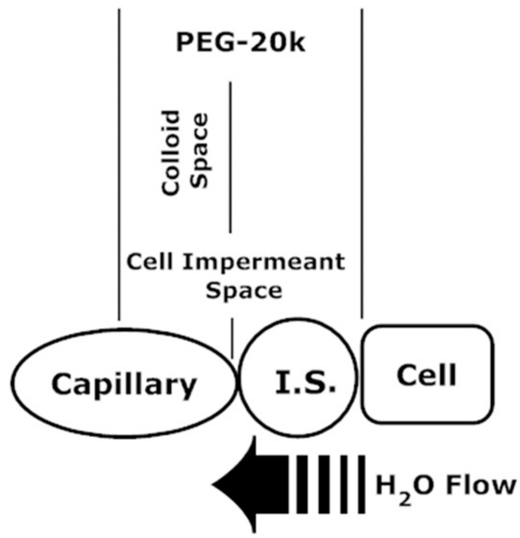


Fig. 1. Hypothetical mechanism of action for PEG-20k in LVR for shock. Cell impermeants can escape the capillary but are too large or charged to enter cells and thus create an osmotic gradient to prevent cell swelling during shock. We hypothesized that PEG-20k acts via biophysical effects on water movement through both cell impermeants and oncotic properties. To test our hypothesis, we tried to recapitulate the PEG-20k effect by combining a cell impermeant (gluconate) with a colloid (albumin). The movement of capillary water with oncotic agents increases capillary pressures that promote capillary flow even under low-volume states. The sum effect is to promote effective and efficient capillary transport and oxygen delivery in the low-volume state. I.S., interstitial space.

Materials and Methods

All animal work was conducted under a protocol approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee, which is governed by the rules and regulations set forth in the National Institutes of Health guide and the United States Department of Agriculture.

Rodent Shock Model. An LVR model was used in adult rats to test the impermeant-based LVR solutions used for prehospital resuscitation during severe hemorrhagic shock (Parrish et al., 2015a,b). Adult male Sprague-Dawley rats were anesthetized and maintained in a light surgical plane of anesthesia with isoflurane during the study. Isoflurane was delivered through a nose cone with a fraction of inspired oxygen of 100%. The animals were allowed spontaneous respirations to control their own ventilation and carbon dioxide levels. Polyethylene catheters were placed in both femoral arteries for blood pressure monitoring and blood sampling, and a third catheter was placed in a femoral vein for fluid administration. Heparin (500 U/kg) was given i.v. to maintain catheter patency. A 1-cm midline incision was created to induce some soft tissue injury and for placement of an intra-abdominal temperature probe. Animals were kept at 38°C using a heating pad and an incandescent light source. Arterial blood pressure, heart rate, and temperature were continuously recorded using PowerLab (AD Instruments, Boston, MA).

After a 15-minute stabilization period, arterial blood was removed at 1 ml/min into a syringe to maintain a mean arterial pressure (MAP) of 30–35 mm Hg. More blood was withdrawn as the animal compensated, but a maximum hemorrhage limit of 60% of blood volume was set. Blood volume (ml) was estimated as weight (g) $\times$ 0.06 + 0.77 as previously described (Arora et al., 2012). A MAP of 30–35 mm Hg was maintained until the plasma lactate reached a value between 9 and 10 mM, as measured every 15 minutes with a handheld lactate analyzer (Lactate Plus, Nova Biomedical, Waltham, MA) and every hour with the ABL-800 blood gas analyzer (Radiometer, Copenhagen, Denmark). Once the target lactate was reached, an LVR equal to 10% of the estimated blood volume was given i.v. over 5 minutes using a syringe infusion pump. Thirty minutes after LVR, serial lactate measurements were taken until the lactate again climbed back to the 9- to 10-mM target because the low volume infusions temporarily lower or stall the accumulation of plasma lactate. The main outcome measured was LVR time, which was defined as the length of time from the start of LVR administration to the time when lactate climbed back to 9–10 mM. The LVR time is a surrogate for tolerance to the low-volume or shock state. It is the length of time that a patient can safely remain in the low-volume state until definitive care and resuscitation are needed, clinically, the "golden hour." At the end of the experiment, the animals were euthanized by Euthasol injection or by exsanguination under anesthesia. The maximum obtainable LVR time in this study was fixed to 240 minutes owing to an isofluorane exposure rule that limited exposure of the animals to that exposure duration. Figure 2 depicts the experimental protocol.

Fluids used for LVR were: 1) normal saline; 2) 15% gluconate in saline, a cell impermeant; 3) 10% albumin in saline; 4) 10% PEG-20k

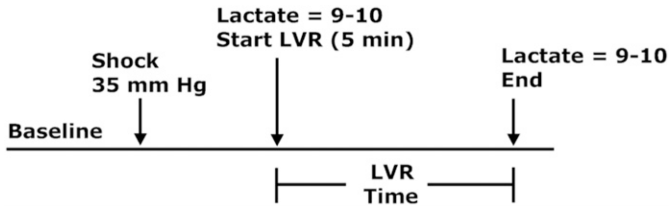


Fig. 2. Timeline diagram of the hemorrhagic shock and resuscitation protocol. Rats were hemorrhaged to a mean arterial pressure of 30–35 mm Hg. Once lactate reached 9 to 10 mM, LVR was administered. The primary outcome was LVR time, which is the length of time from start of LVR to the time at which lactate climbed back to 9 to 10 mM.

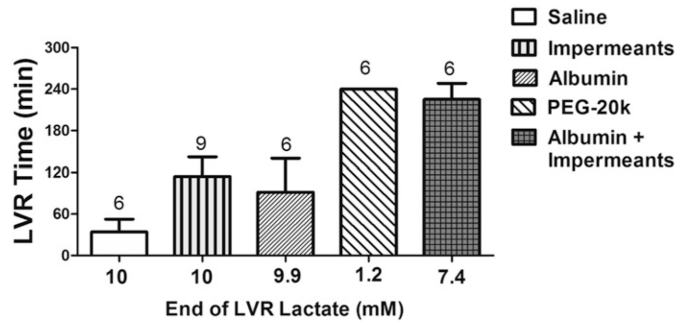


Fig. 3. The effect of different LVR solutions on LVR time. Data are presented as mean (S.D.). Numbers below bars indicate mean lactate at the end of LVR time, which by definition should be 9 to 10 mM. Numbers above bars indicate sample size. All treatment groups had significantly higher LVR times than the saline (control) group. No significant difference was seen between PEG-20k and the albumin + impermeant group.

in saline; and 5) 15% gluconate + 10% albumin in saline. Other outcomes recorded included the lactate at the end of the LVR time, which in most cases was 9 to 10 mM by definition. MAP was also recorded throughout the experiment. Test agents used for i.v. resuscitation (sodium gluconate, bovine albumin, and PEG-20k were obtained from Sigma-Aldrich, St. Louis, MO).

Regional Blood Flow. In another series of studies (n = 6), local capillary blood flow was studied using the colored microsphere technique (Adams et al., 2001; Parrish et al., 2015a). Animals were prepared as previously described, but a catheter was also placed into the aortic root using real-time pressure and pressure waveforms as indicators of catheter tip location by identifying the aortic valve. During the stabilization/baseline period, 300-μl colored microspheres (Triton Technologies, San Diego, CA) were rapidly injected into the aortic root as a calibrated arterial reference blood sample was simultaneously removed from the femoral artery catheter with a withdrawal pump at a constant rate of 0.25 ml/min. A different colored microsphere was injected 30 minutes after LVR. After the study, tissue samples were removed from the major organs, and microspheres were recovered from the tissue samples and reference arterial blood samples by alkaline digestion and repeated centrifugations. Dye coating purified colored microspheres was extracted with acidified 2-ethoxyethyl acetate and quantitated using a UV-visible spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). Individual colors were resolved using a matrix inversion algorithm from the composite spectra. Blood flow was calculated by the tissue dye content using the reference blood draw as a blood flow standard. Correction for microsphere loss occurred using the recovery of blue microspheres that were added to the tissues as an internal standard before digestion (10,000 spheres added per sample). All flows were normalized to 100 g of tissue weight and expressed as the change from baseline values before shock and LVR.

Blood-Volume Determinations. Total blood volume of rats after shock and after various times after LVR was calculated using the indicator dilution technique (Iijima et al., 1998; Ertl et al., 2007). An i.v. bolus of fluorescein isothiocyanate (FITC)-albumin (Sigma-Aldrich, St. Louis, MO) of known volume and activity was administered and, a reference i.v. sample was taken 15 minutes later for estimation of the volume dilution effect. Plasma volume was calculated by the degree of FITC dilution using a standard dilution curve with saline. Plasma volume was divided by 1-hematocrit to determine the circulating blood volume. Blood volume was also assessed by the same indicator dilution principle using hematocrit during the LVR period. The assumptions of this method were: 1) that the red blood cells stay in the vascular compartment during LVR; 2) that the volume of the packed cell component remains constant during LVR (because no further bleeding is allowed); and; 3) that changes in hematocrit during LVR are inversely proportional to changes in the plasma volume component of the intravascular space. Baseline blood volumes before shock were estimated using a formula as previously described (Parrish et al., 2015a).

Circulating Cytokines. To determine a possible role for early type 1 T-helper cytokines in this model, we measured plasma levels of interleukin-1β (IL-1β) and tumor necrosis factor-α because they are representative of early type 1 T-helper cytokines that may be formed during hypovolemic shock and early resuscitation injury and play a role in later autolytic inflammation (Sato et al., 2008). Cytokines from plasma samples were determined by standard enzyme-linked immunosorbent assay using commercially available kits (Boster Bio, Pleasanton, CA).

Statistical Analysis. Data are expressed as mean ± S.D. Each group consisted of five to nine rats, which was derived from power analysis and the known variance of the data from similar studies. Data were analyzed by one-way or two-way analysis of variance and Bonferroni multiple comparison correction using the InStat program (GraphPad Software, Inc., La Jolla, CA). A P-value < 0.05 was considered statistically significant.

Results

The effects of a variety of chosen LVR solutions on LVR time are shown in Fig. 3. Normal saline was the control LVR fluid, which produced an LVR time of 34 ± 8 minutes. The LVR time significantly increased to 114 ± 10 minutes and 92 ± 20 minutes in the gluconate and albumin groups, respectively, compared with saline. The LVR time for the PEG-20k group was 240 ± 0 minutes ($P < 0.05$, relative to saline, gluconate, and albumin groups). This LVR time was cut off for technical reasons and would have been higher because the lactate at 240 minutes was only 1.2 mM, which is well below the target cutoff of 9 to 10 mM. No significant difference in LVR times was found between PEG-20k and the albumin + gluconate–treated groups (240 ± 0 minutes and 225 ± 24 minutes, respectively). The true comparison between these two groups is unknown, however, because the full LVR time in the PEG-20k group was not realized. This occurred because the lactate target was not reached owing to the duration of exposure of isofluorane anesthesia (i.e., 240 minutes). Technically, this result was attributable to time-dependent anesthesia problems in the animals after 4 hours. Therefore, the reported magnitude of the PEG-20k effect during shock in Fig. 3 is underestimated when measured by the LVR time.

Similar to the LVR times, the MAPs in the impermeant, PEG-20k, and albumin groups were much higher throughout the LVR period compared with the saline LVR control (Fig. 4). Generally, the MAPs during LVR correlated with the LVR times such that the groups with the longest LVR time (PEG-20k) also had the highest MAP and vice versa (Fig. 4).

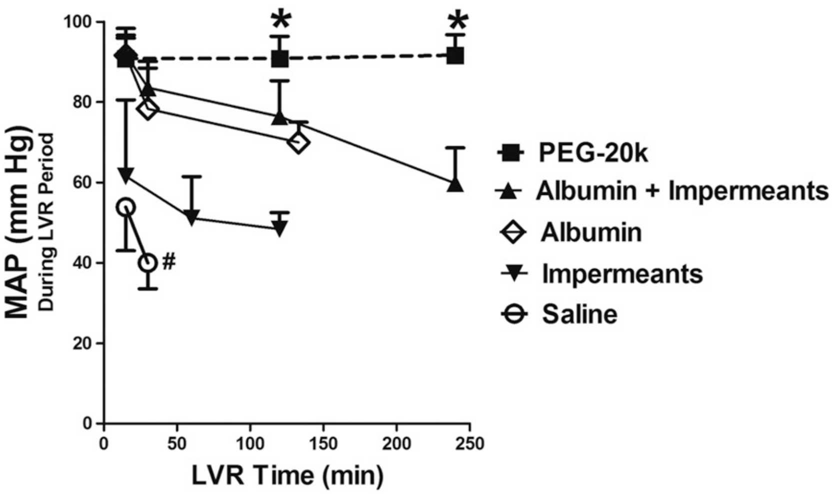


Fig. 4. MAPs after LVR administration, measured at 15 minutes, 30 minutes, throughout the LVR period, and at end of LVR time. $^*P < 0.05$ relative to the other values at the same corresponding times in the other groups. $^{\#}P < 0.05$ relative to all other values.

Reductions in capillary blood flow after shock and LVR, as measured by the colored microsphere technique, were significantly less in all organs and tissues (except the ileum) during the LVR period in PEG-20k resuscitated animals relative to the saline-resuscitated control animals (Fig. 5). All flow values (except in the left ventricle) in the saline group after LVR were statistically lower than their paired baseline values, and all flow values in the PEG-20k group after LVR were statistically unchanged from their paired baseline values. In other words, shock and LVR with saline caused significant reductions in local tissue blood flow, which was prevented when PEG-20k was used as the LVR solution.

Blood volume measurements made after hemorrhage and at 15, 30, and 60 minutes after LVR administration in the saline and PEG-20k groups are shown in Fig. 6. Blood volume was estimated before shock. Shock significantly reduced blood volume in both groups relative to baseline. Resuscitation with PEG-20k significantly increased blood volume at all times after LVR compared with the saline control LVR solution using either indicator dilution technique. Resuscitation with low volumes of 10% PEG-20k, but not saline, caused blood volume to increase significantly above values observed after hemorrhage.

Plasma levels of IL-1β and tumor necrosis factor-α at baseline, after hemorrhage, and 60 minutes after LVR are shown in Fig. 7. IL-1β concentrations after saline LVR were statistically higher compared with the corresponding levels after PEG-20k LVR. All other values were not significantly different either during the shock protocol for either cytokine or between treatment groups for any corresponding time points during the protocol for either cytokine.

Discussion

Our previous studies have demonstrated the efficacy of a novel platform of LVR crystalloid solutions in extending tolerance to the low-volume state or the amount of time that a severely shocked patient can safely remain in the low-volume state until definitive resuscitation and medical care are delivered. These solutions contain cell impermeants and are designed specifically to reduce the amount of ischemia-induced cell swelling during shock. LVR solutions that used the specific polymer PEG-20k produced striking biologic effects that increased the tolerance to the low-volume state

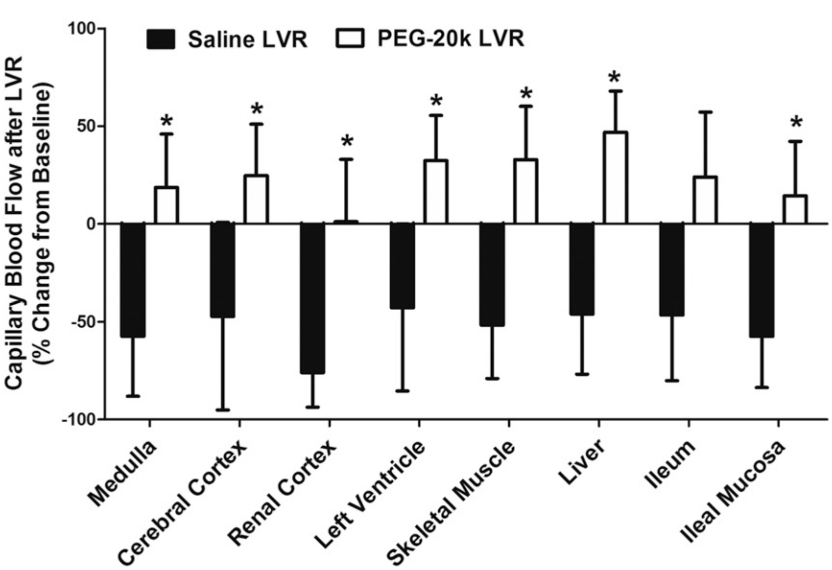


Fig. 5. Capillary blood flow measured 30 minutes after LVR. Data are presented as mean (S.D.) as percentage of change from baseline. Each rat served as its own baseline. $^*P < 0.05$ compared with the saline group. For the saline group, all flows except left ventricle were statistically less than paired baseline flows. For PEG-20k, all flows were not different from baseline. $n = 5$.

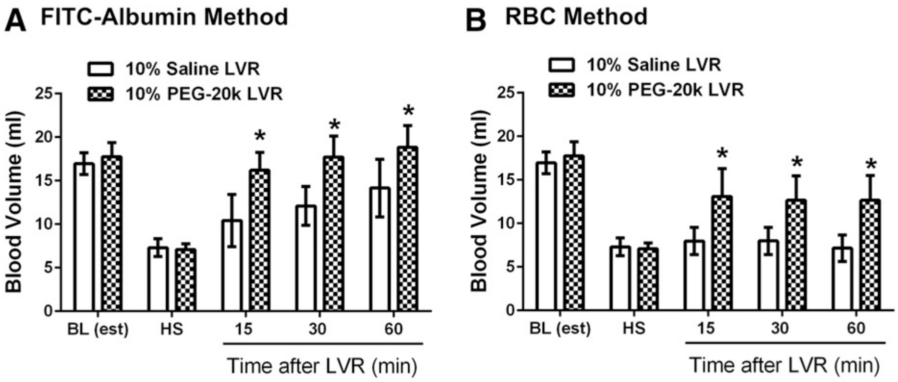


Fig. 6. Circulating blood volume measured in rats by the indicator dilution technique using a FITC-labeled albumin probe (A) or hematocrit (B) to estimate the size of the intravascular fluid compartment during LVR with saline or saline containing 10% PEG-20k. Blood volume values were estimated in the rats before shock (BL, baseline), and measured with indicator dilution after the hemorrhagic shock (HS) period, and after the LVR period. Values are mean ± S.D. for four rats in each group. $^{*}P < 0.05$ relative to the corresponding value in the saline group. Both LVR solutions were given at a volume of 10% of the estimated baseline blood volume. Saline was 0.9% NaCl solution, and PEG-20k was a 10% wt/vol solution of polyethylene glycol (mean mol. wt. = 20,000 Da) dissolved in saline.

(LVR time) 4- to 8-fold over previous classes of smaller cell impermeants or conventional saline-based resuscitation fluids. The objective of this study was to explore the mechanisms of action of PEG-20k that account for these strong biologic and preclinical effects.

Simple cell-impermeant molecules, like gluconate, trehalose, and raffinose, have been used in organ-preservation solutions to prevent tissues from swelling in cold ischemic environments. Ideal cell impermeants are molecules that have a unique size enabling them to freely escape the capillary space but not cross the cell membrane because they are too large or charged. Thus, they accumulate outside the cell and osmotically hold water from entering the cell, which is its normal propensity during shock and ischemia when energy-dependent volume-control mechanisms fail (Na^+/K^+ ATPase). These simple impermeants prevented cell and tissue swelling in rodent models of hemorrhagic shock when introduced into LVR solutions. Their low toxicity and chemical inertness allow them to be used successfully in high concentrations capable of exerting these necessary biophysical effects on water shifts during shock. Gluconate quadrupled the LVR time compared with saline. In an attempt to optimize this effect, we added colloidal molecules to create a second osmotic gradient in the microcirculation, which was designed to pull water into the capillary space. The first studies used PEG-20k as a colloid, together with the simple impermeant gluconate. This increased the LVR time 7-fold in the rodent model, which suggested that the double-gradient approach may have worked; however, subtraction experiments using only PEG-20k without the gluconate produced the same effect as the two together. It was then hypothesized that the larger PEG-20k molecule may be acting as both a cell impermeant and a colloidal molecule.

Further studies indeed determined that this was true since the osmotic reflection coefficient for PEG-20k was determined to be 0.5 in the rat microcirculation (thoracic and mesenteric beds) under nonshock conditions (Parrish et al., 2015b). This means that roughly a third of the PEG-20k escapes the capillary space to load into the interstitium, where it acts like a simple cell impermeant (gluconate) and two-thirds of the molecules in the circulation remains behind in the capillary space, where it acts as a colloidal agent to produce the second osmotic gradient. Although this double-gradient effect of PEG-20k is completely and unambiguously supported by the biophysical osmotic reflection coefficient data, the translation of these properties into the strong biologic effects seen with PEG-20k remains less certain. To make this biologic link and support the hypothesis that the second osmotic gradient indeed contributes to the very long LVR time of PEG-20k during shock, we attempted to recapitulate the biologic effect of PEG-20k with two distinct molecules used together: 1) a small ideal cell impermeant (gluconate) that produces an osmotic gradient between the intracellular and the extracellular space and 2) a classic colloidal agent (albumin) that produces a second osmotic gradient between the interstitial space and the capillary (intravascular) space.

Indeed, the use of these two distinct molecules (albumin and gluconate) produced a biologic effect during LVR that was similar to that when PEG-20k was used alone. This result supports the hypothesis that the biologic effect seen with PEG-20k may be due, in part, to its unique biophysical attributes that allow it to behave as both an ideal impermeant and a colloid in the microcirculation during shock states; however, since we do not know the exact LVR time with PEG-20k alone, because the period was terminated early, biologic differences between this group and the albumin + gluconate

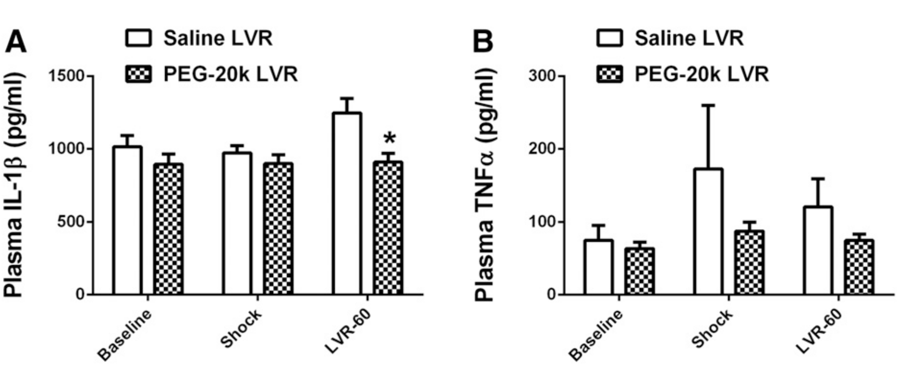


Fig. 7. Plasma IL-1β (A) and tumor necrosis factor-α (B) concentrations in rats at baseline after hemorrhagic shock and after 60 minutes of LVR (LVR-60). Rats received either a saline LVR or a PEG-20k LVR after shock equal to 10% of the calculated blood volume administered over 10 minutes. All values are mean ±S.D., n = 6. $^{*}P < 0.05$ relative to the corresponding saline value.

group probably do exist, which suggests that not all the PEG-20k effect may be attributable to osmotic gradients. Whereas other biologic effects of PEG-20k are likely in these settings, they currently remain unknown. Protection and hydration of the shock-eroded glycocalyx and induction of cell-surface immunocamouflage by PEG-20k polymers remain strong possibilities.

Rats receiving saline had a transient increase in MAP during LVR infusion, but the pressure started to drop as soon as the infusion stopped. This happens because saline physiologically distributes unequally between the vascular and interstitial compartments. About 20% of the administered saline volume will remain in the intravascular space, and 80% goes elsewhere (Haupt, 1986, 1989). Since only the 20% remaining in the capillaries supports arterial pressure, it is not surprising that saline given in low volumes during shock produce poor or absent effects on the arterial pressure. When PEG-20k–based LVR solutions are administered, it may prevent saline from loading into the interstitium while simultaneously returning the fluid that leaked into the interstitium back into the capillary spaces during the shock period (Gosling, 2003; Keel and Trentz, 2005; Kumar et al., 2010). These passive-volume shifts reduce local tissue swelling, decompress the microcirculation, reduce resistance to flow, and reload the capillaries. All these changes drive local tissue perfusion and provide cardiac preload as demonstrated directly by the increase in MAP and indirectly by the rapid clearance of lactate in LVR and by direct measurements of increased capillary blood flow with PEG-20k compared with saline. This is further supported by the significant effects of PEG-20k solutions on expansion of the plasma and blood volumes after their administration during the LVR period. PEG-20k maintenance of perfusion pressure and lactate clearance in the low-volume state can extend up to 8 hours (unpublished data), which was the longest period examined so far.

Hemorrhagic shock decreases oxygen delivery, which results in the accumulation of oxygen debt during the low-volume state. Using lactate clearance directly and the LVR time indirectly, which relies on lactate levels, we were able to demonstrate clearly that PEG-20k–based LVR solutions both stop accumulation of oxygen debt and rapidly repay the debt, even during the low-volume state. This is supported by the rapid drop in lactate levels after PEG-20k–based LVR and the extremely long LVR times relative to the values seen with conventional saline LVR. Whereas about 50% of this drop in lactate can be attributable to dilution from an expanding intravascular volume, much of the remaining lactate clearance may be due to increased efficiency of microvascular capillary oxygen transfer and/or an overall increase in oxygen delivery, which drives the conversion of lactate back to pyruvate for subsequent aerobic ATP synthesis. In fact, preliminary studies in a large animal porcine model of shock and LVR indicate that PEG-20k–based LVR under similar low-volume conditions leads to a hyperdynamic cardiovascular response characterized by cardiac output increasing 50% higher than preshock baseline values over much of the low-volume state (Plant et al., 2016). These combined factors likely account for the apparent rapid oxygen debt repayment and the 100% overnight survival (Parrish et al., 2015a) seen in PEG-20k–treated rodents. In patients with long prehospital transport times, this can limit further ischemic and reperfusion injury and possibly begin debt repayment during the transport period.

A likely mechanism of action of PEG polymers in LVR not addressed by this study includes reconstruction of the endothelial glycocalyx by PEG-20k. Shock and crystalloid resuscitation are known to erode the glycocalyx, thus promoting resuscitation injury by promoting cellular inflammation (Torres Filho et al., 2013; Torres et al., 2013). Polyethylene glycol polymers are known to bind to the cell membrane with their accompanying water layers (Neu et al., 2003) that could effectively rebuild the glycocalyx during the LVR and reperfusion period (Hauet and Eugene, 2008). Although this likely happens, it seems that such effects would be expressed after longer periods of resuscitation since cellular inflammation may require hours rather than minutes, which is the time period where rapid capillary blood flow and lactate clearance were observed with PEG-20k in this study. It is reasonable to suggest that PEG-20k LVR may reload peripheral capillaries by early osmotic water transfer while having later effects on glycocalyx-mediated cellular inflammation. Our cytokine data also support this.

In conclusion, 10% PEG-20k is a novel LVR fluid with encouraging potential for prehospital use in hemorrhagic shock. By improving local oxygen delivery and capillary perfusion, these solutions increase tolerance to the shock state during prolonged prehospital and transport periods. This study tests a likely biophysical mechanism for its efficacy, namely, the osmotic cell-to-capillary transfer of accumulated water that drives efficient local perfusion under low-volume conditions.

Authorship Contributions

Participated in the research design: Plant, Parrish, Limkemann, Mangino.

Conducted Experiments: Plant, Parrish, Limkemann, Mangino.

Performed Data Analysis: Plant, Mangino.

Wrote or contributed to the writing of the manuscript: Plant, Parrish, Limkemann, Ferrada, Aboutanos, Mangino.

References

Adams JA, Mangino MJ, Bassuk J, Kurlansky P, and Sackner MA (2001) Regional blood flow during periodic acceleration. *Crit Care Med* **29**:1983–1988.

Arora TK, Malhotra AK, Ivatury R, and Mangino MJ (2012) L-arginine infusion during resuscitation for hemorrhagic shock: impact and mechanism. *J Trauma Acute Care Surg* **72**:397–402.

Chaudry IH, Sayeed MM, and Baue AE (1974) Depletion and restoration of tissue ATP in hemorrhagic shock. *Arch Surg* **108**:208–211.

Cotton BA, Guy JS, Morris, Jr JA, and Abumrad NN (2006) The cellular, metabolic, and systemic consequences of aggressive fluid resuscitation strategies. *Shock* **26**: 115–121.

Duchesne JC, McSwain, Jr NE, Cotton BA, Hunt JP, Dellavolpe J, Lafaro K, Marr AB, Gonzalez EA, Phelan HA, Bilski T, et al. (2010) Damage control resuscitation: the new face of damage control. *J Trauma* **69**:976–990.

Ertl AC, Diedrich A, and Raj SR (2007) Techniques used for the determination of blood volume. *Am J Med Sci* **334**:32–36.

Gosling P (2003) Salt of the earth or a drop in the ocean? A pathophysiological approach to fluid resuscitation. *Emerg Med J* **20**:306–315.

Hauet T and Eugene M (2008) A new approach in organ preservation: potential role of new polymers. *Kidney Int* **74**:998–1003.

Haupt MT (1986) Colloidal and crystalloidal fluid resuscitation in shock associated with increased capillary permeability. *Curr Stud Hematol Blood Transfus* **53**: 86–100.

Haupt MT (1989) The use of crystalloidal and colloidal solutions for volume replacement in hypovolemic shock. *Crit Rev Clin Lab Sci* **27**:1–26.

Iijima T, Iwao Y, and Sankawa H (1998) Circulating blood volume measured by pulse dye-densitometry: comparison with (131)I-HSA analysis. *Anesthesiology* **89**: 1329–1335.

Keel M and Trentz O (2005) Pathophysiology of polytrauma. *Injury* **36**:691–709.

Kloner RA (2011) No-reflow phenomenon: maintaining vascular integrity. *J Cardiovasc Pharmacol Ther* **16**:244–250.

Kumar V, Abbas A, Fausto N, and Aster J (2010) *Pathologic Basis of Disease*, Saunders Elsevier, Philadelphia.

Mees N, Southard JH, and Belzer FO (1982) Inhibition of ischemic induced cellular swelling in kidney cortex tissue by lactobionate anions. *J Trauma* **22**: 118–120.
Neu B, Armstrong JK, Fisher TC, and Meiselman HJ (2003) Surface characterization of poly(ethylene glycol) coated human red blood cells by particle electrophoresis. *Biorheology* **40**:477–487.
Parrish D, Lindell SL, Reichstetter H, Aboutanos M, and Mangino MJ (2016) Cell impermeant-based low-volume resuscitation in hemorrhagic shock: a biological basis for injury involving cell swelling. **263**(3):565–572.
Parrish D, Plant V, Lindell SL, Limkemann A, Reichstetter H, Aboutanos M, and Mangino MJ (2015b) New low-volume resuscitation solutions containing PEG-20k. *J Trauma Acute Care Surg* **79**:22–29.
Plant V, Limkemann A, Liebrecht L, Blocher C, Ferrada P, Aboutanos M, and Mangino MJ (2016) Low-volume resuscitation using polyethylene glycol-20k in a preclinical porcine model of hemorrhagic shock. *J Trauma Acute Care Surg* **81**: 1056–1062.
Reffelmann T and Kloner RA (2002) The “no-reflow” phenomenon: basic science and clinical correlates. *Heart* **87**:162–168.
Rezkalla SH and Kloner RA (2002) No-reflow phenomenon. *Circulation* **105**:656–662.
Riha GM, Kunio NR, Van PY, Hamilton GJ, Anderson R, Differding JA, and Schreiber MA (2011) Hextend and 7.5% hypertonic saline with Dextran are equivalent to lactated Ringer’s in a swine model of initial resuscitation of uncontrolled hemorrhagic shock. *J Trauma* **71**:1755–1760.
Riha GM, Kunio NR, Van PY, Kremenevskiy I, Anderson R, Hamilton GJ, Differding JA, and Schreiber MA (2013) Uncontrolled hemorrhagic shock results in a hypercoagulable state modulated by initial fluid resuscitation regimens. *J Trauma Acute Care Surg* **75**:129–134.
Sato H, Kasai K, Tanaka T, Kita T, and Tanaka N (2008) Role of tumor necrosis factor-alpha and interleukin-1beta on lung dysfunction following hemorrhagic shock in rats. *Med Sci Monit* **14**:BR79–BR87.
Southard JH and Belzer FO (1980) Control of canine kidney cortex slice volume and ion distribution at hypothermia by impermeable anions. *Cryobiology* **17**:540–548.
Stein DM and Scalea TM (2012) Capillary leak syndrome in trauma: what is it and what are the consequences? *Adv Surg* **46**:237–253.
Torres Filho I, Torres LN, Sondeen JL, Polykratis IA, and Dubick MA (2013) In vivo evaluation of venular glycocalyx during hemorrhagic shock in rats using intravital microscopy. *Microvasc Res* **85**:128–133.
Torres LN, Sondeen JL, Ji L, Dubick MA, and Torres Filho I (2013) Evaluation of resuscitation fluids on endothelial glycocalyx, venular blood flow, and coagulation function after hemorrhagic shock in rats. *J Trauma Acute Care Surg* **75**:759–766.
van Lambalgen AA, van den Bos GC, and Thijs LG (1990) Whole body plasma extravasation in saline and Haemaccel loaded rats: effects of endotoxemia. *Int J Microcirc Clin Exp* **9**:303–318.

Address correspondence to: Dr. Martin J. Mangino, Virginia Commonwealth University, Medical College of Virginia Campus Department of Surgery, 1101 E. Marshall St. Richmond, VA 23298. E-mail: mjmangino@vcu.edu

Thromboelastographic Analysis of Novel Polyethylene Glycol Based Low Volume Resuscitation Solutions

Loren K. Liebrecht, M.D.[1], Jason Newton, Ph.D.[2] Erika J. Martin, M.S.[5], Nina Wickramaratne, M.D.[1], Sudha Jayaraman, M.D.[1], Jinfeng Han, B.S.N. [1], Michel Aboutanos, M.D.[1], Donald F. Brophy, Pharm.D.[5], and Martin J. Mangino, Ph.D.[1,3,4]

[1]Department of Surgery, Division of Acute Care Surgery, [2]Department of Biochemistry and Molecular Biology, [3]Department of Physiology and Biophysics, [4]Department of Emergency Medicine, School of Medicine, and [5]Department of Pharmacotherapy and Outcomes Science, School of Pharmacy, Virginia Commonwealth University, Richmond, VA 23298

Corresponding Author
Martin J. Mangino
Department of Surgery
Virginia Commonwealth University
School of Medicine
1101 E. Marshall St.
Richmond, VA 23298
804-628-3226
804-828-8300 (FAX)
mjmangino@vcu.edu

Support: This work was supported by grants from the Department of Defense W81XWH-16-2-0040 and W81XWH-12-1-0599 to Dr. Mangino

Running Head: Clot formation after polymer based crystalloids

ABSTRACT

Low volume resuscitation (LVR) in shock prevents deleterious effects of crystalloid loading in pre-hospital settings. Polyethylene glycol 20,000 (PEG-20k) based LVR solutions are 20-fold more effective at maintaining perfusion and survival in the low volume state compared to conventional crystalloids. The aim of this study was to examine the effects of PEG based LVR solutions on platelet and coagulation function of whole blood. Citrated blood from volunteers (n=26) or early admission severely injured trauma patients (n=12) were diluted 10% with various LVR solutions in a matched design with a paired volume control and studied using thromboelastography (TEG). In healthy volunteers and patients, 10% PEG-20k significantly increased clot amplification time (k), decreased propagation (angle), maximal clot size and strength (MA), and the overall coagulation index (CI), but not clot initiation (R) or fibrinolysis (Ly30), relative to paired saline dilutional controls. Clinically, K, angle, and MA were just outside of the normal limits in volunteers but not in patients. No statistical differences existed between PEG-20k and Hespan (HES) in either volunteers or trauma patients. In a dose response series in volunteer blood, effects of 10% PEG-20k on TEG were reversed and normalized by lower concentrations (7.5% and 5%). Furthermore, 7.5% PEG-20k produced similar resuscitation effects as 10% in shock models. In conclusion, PEG-20k based LVR solutions produced a dose-dependent minor hypocoagulative state, possibly associated with changes in clot propagation and platelet function, which can be reversed by dose reduction in concentration while providing superior LVR, microvascular rescue, and lactate clearance.

INTRODUCTION

Minimizing the use of crystalloids and utilizing blood products after trauma are now becoming mainstream in civilian trauma centers. Damage control resuscitation is also emerging as standard of care for the US Department of Defense, according to the Joint Theater Trauma Systems Clinical Practice Guidelines (JTTS CPG). When blood products are not available for resuscitation, crystalloid solutions are administered. However, only a fraction of infused crystalloid volume stays in the intravascular space and the use of low volume crystalloids has minimal effects on pressure and perfusion (1, 2). The movement of crystalloid fluid from capillary to interstitium is compounded by the increase in capillary permeability from trauma-related inflammation and trauma-induced capillary leak syndrome (TICS) (3). Furthermore, crystalloid resuscitation exacerbates TICS, acidosis, hypothermia, and coagulopathy (3, 4). Other resuscitation solutions such as hypertonic saline or starch have had disappointing results (5, 6) including concerns and risks associated with their use (4, 7). There remains a need for a better crystalloid fluid that can be given at a low volume to resuscitate patients in severe hemorrhagic shock awaiting definitive treatment, especially for the prehospital setting.

Recently, polyethylene glycol polymers of specific molecular weight ranges have been used in crystalloid solutions to act as highly effective Low volume Resuscitation (LVR) solutions (2, 8-10). These polymers non-energetically move isotonic fluid from intracellular and interstitial spaces into the capillary space by simple osmotic actions in response to metabolic cell swelling that occurs in shocked and ischemic tissues. As water flow moves from the interstitial spaces to the capillaries, the capillary exchange in the tissues dramatically improves under very low volume conditions because the microcirculation is decompressed while the capillary spaces are re-loaded with volume and pressure for driving flow (10). This causes rapid clearance of lactate, increased blood pressure, and tolerance to the low volume state (8). While these polymers work several fold better than hydroxyethyl starch based

polymers, implying different mechanisms of action, interference with blood clotting and coagulation may be shared by both types of polymers. Intravenous admiration of Hextend and Hespan are complicated by both renal toxicity and coagulopathies (11), which in trauma settings are a concern. Therefore, the purpose of this study was to examine any possible effects of PEG-20k based LVR solutions on whole blood coagulation and platelet function and to identify any existing mechanisms of such coagulopathies.

METHODS:

Low Volume Resuscitation (LVR) solutions: As a volume control for all experimental solutions, 0.9% NaCl (normal saline, NS) was prepared using 9 g/L sodium chloride. This solution served both as a volume control since other solutions were dissolved in similar vehicles and served as a volume dilution control to account for the 10% volume that was administered in all LVR settings. A military medicine control used a 6% hydroxyethyl starch (HES) solution and was purchased from the manufacturer under product name HESPAN®, formulated with 6% hetastarch [molecular weight (MW) ~600 kDa (range 450-800 kDa) with ~0.75 molar substitution at primarily the C-2 glucose unit] in 0.9% sodium chloride. The experimental solutions consisted of polyethylene glycol (PEG) 20,000 mw (PEG-20k) dissolved in normal saline at concentrations of 10%, 7.5%, and 5%. Polyethylene Glycol-20k was purchased from Sigma Chemical Co (St. Louis, MO) as the molecular biology grade material. All solutions were either prepared fresh or filter sterilized using 0.22 micron filtration for storage in polypropylene containers to exclude bacterial degradation.

Preparation of Blood and TEG Assay: An internally matched comparative analysis was designed where each study participant would serve as their own control. Each enrolled study participant's blood was diluted 10% with various resuscitative fluids, always including a saline dilution control paired with 6% HES, and 10% PEG-20k. A dose-response series was also conducted using PEG-20k at concentrations of 5%, 7.5%, and10% in saline at the same 10% dilution with whole blood. Both healthy volunteers and trauma patients were enrolled under an approved VCU IRB protocol. Healthy volunteers were without comorbidities or any medications and between 18-50 years of age. Trauma patients were those arriving at the highest alert level to our trauma system with systolic blood pressure below 95 mm Hg and plasma lactate levels greater than 4.6 mM. Trauma patient blood was collected early after arrival, usually within 30 minutes before they received significant blood or fluid transfusions. The goal

was to select only those patients that received only small amounts (0-300 ml) of saline or LR crystalloid in the field or en-route to the emergency department. Patients who received larger fluid volumes or blood products were excluded from the study.

Venous blood samples from individual healthy volunteers or from trauma patients were drawn into citrate treated vacutainer tubes (15 ml total), pooled, and diluted 10% with saline, 6% Hextend, 10% PEG-20k, 7.5% PEG-20k, or 5% PEG-20k, gently mixed by inversion, and analyzed on a TEG-5000 thromboelastograph (Haemonetics Corp.) within 2 hours from blood draw. The TEG data were reported as six outcome parameters that describe different functional attributes of the clotting and coagulation system of whole blood under these conditions. These include: **R**, a measure of the time to initiate fibrin clot formation; **K**, time to achieve a predetermined clot size and strength (20 mm clot size). This represents amplification of the clotting cascade; **Alpha (α)** or angle of the slope between R and k, which characterizes the propagation phase and thrombin burst converting fibrinogen into fibrin with fibrin cross linking; **MA**, the maximum amplitude of the clot that represents clot strength, which is generally composed of 80% platelet and 20% fibrin responses; **LY30,** the % lysis of the clot 30 min after maximal formation (MA), which represents rates of fibrinolysis of the clot; and **CI**, the coagulation index that is a mathematical model of overall coagulation responses using the other TEG parameters. Blood clot initiation in the TEG was started by a combination of re-calcification and kaolin activation.

Shock Resuscitation Testing: To test resuscitation outcomes of LVR solutions specifically for comparison with TEG outcomes in the dose response series of experiments, a standard rat lactate controlled model of severe hemorrhagic shock with low volume resuscitation was used as previously described in great detail elsewhere (2, 9, 10). Briefly, we determined tolerance to the low volume state in severely shocked acutely anesthetized rats. Arterial bleeding to a mean arterial pressure of 35 mmHg was maintained until plasma lactate rose to 9-10 mM, which initiated low volume resuscitation using

saline control, or 10% and 7.5% PEG-20k solution, all given intravenously at a volume equal to 10% of the estimated blood volume of the rat (12). Immediately after LVR solutions are given, lactate falls but then begins to rise again until it again reaches the 9-10 mM limit. The time from the start of LVR infusion until the lactate rises back to its limit again (9-10 mM) is recorded as the LVR time. The LVR time and the lactate and MAP values at the end of the LVR time are all outcome measures of the tolerance to the hypotensive state.

Statistical Analysis: All statistical analyses were performing using GraphPad Prism version 6.07 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Data groups were analyzed for outliers using the nonlinear regression ROUT method with Q=1%, the maximum desired false discovery rate. Normality of Gaussian distribution was then assessed using the D'Agnostino-Pearson ombinus K2 method. Most data were then analyzed by the non-parametric ANOVA Kruskal-Wallis test with the Mann-Whiteney U test for multiple comparisons of means. Most data are expressed as the median with the 2nd and 3rd interquartile ranges and the upper and lower extremes (box and whiskers).

RESULTS:

Initiation of Clotting: The TEG R time is shown in Figure 1. For healthy volunteers, initiation times were lower than the normal range for both saline and HES diluted whole blood and just within normal in PEG diluted blood. PEG significantly lengthened the initiation time, relative to the saline dilutional controls. Similar trends were seen in blood from trauma patients where all of the R times were below normal. For PEG dose responses, all values were below normal irrespective of PEG concentration ranges from 5-10%. The volunteers and patients showed similar R times for all groups.

Amplification of Clotting: The TEG K parameter is shown in figure 2 for the volunteers, the trauma patients, and a PEG dose response series of diluted blood. The trend in all groups was a significant increase in the amplification time in both HES and PEG diluted whole blood. This occurred in blood from healthy volunteers or blood obtained from trauma patients and the results were essentially the same. The elevated K times in the 10% PEG diluted blood from volunteers was brought back into the normal range by dose dependently decreasing the concentration of PEG in the LVR solution to 7.5% and 5%.

Clot Propagation: Figure 3 shows the data for the TEG angle parameter in volunteers, trauma patients, and blood in the PEG dose-response study in health volunteer blood. The angle significantly decreases in both HES and PEG diluted blod with larger decreases in PEG groups. This effect is qualitatively and quantitatively the same in blood obtained from both volunteers and trauma patients. The significant decrease in the angle or propagation rate by 10% PEG-20k was normalized by reducing the concentration to 7.5% and 5% in the LVR solutions.

Maximum Clot Strength: The TEG MA parameter is shown in Figure 4 for blood from healthy volunteers, in blood from trauma patients, and volunteer blood in a PEG-20k dose-response series. The maximum strength or clot size is generally believed to represent a contribution by both platelets (80%) and fibrin (20%) under these conditions. The MA response was significantly reduced in both HES and PEG diluted blood, relative to the saline dilutional controls, in blood obtained from both healthy volunteers and trauma patients. The patterns were almost identical in volunteers and trauma patients. The significantly lower clot strength in blood diluted with 10% PEG-20k could be dose-dependently reversed by progressively lowering the PEG concentration in the LVR solutions to 7.5% and 5%.

Clot Lysis: The TEG Ly30 data are provided in Figure 5. The rate of clot lysis was mostly less than 1-2% after 30 minutes and was not affected by the dilution with any LVR solution, including PEG-20k. The rate of fibrinolysis was also not different in the trauma patients compared to healthy volunteers.

Coagulation Index: The coagulation index is shown in figure 6 for the blood dilutions in the healthy volunteers, the trauma patients, and the volunteers in the PEG-20k dose-response study. The coagulation index (CI) is a mathematical compilation of other TEG parameters and is described by $CI= -0.3258R-0.1886K+0.1224MA+0.0759\alpha-7.7922$. The normal range for CI is between 3.0 and -3.0. As shown in Figure 6, blood from either healthy volunteers or trauma patients had significant reductions in the CI with HES or PEG-20k but the larger response was seen with PEG-20k at 10% concentration. The CI could be dose dependently reversed into the normal range by reductions in the concentration of the PEG-20k in the LVR solution.

Resuscitation Performance of 7.5% PEG-20k Solutions: Because slight reductions in the concentration of PEG-20k in LVR solutions caused a normalization of the TEG parameters, relative to those observed using 10% PEG, the effects of the reduced concentration on resuscitation outcomes was determined in our common rodent hemorrhagic shock model. Figure 7 clearly shows that reducing the concentration of PEG-20k from 10% to 7.5% produces an equivalent resuscitation effect to the one observed with 10% PEG-20k. This is true when the quality of the resuscitation is described by the LVR time (panel A), the terminal lactate values (panel B), or the terminal mean arterial blood pressures (panel C).

DISCUSSION:

Low volume crystalloid resuscitation is used in early pre-hospital resuscitation of severely shocked patients in civilian and military settings for two important reasons. First because lower dilutional volume replacement has superior outcomes compared to the traditional large volume resuscitation strategies (11) and second, because low volume crystalloid is friendly to resource poor austere environments of distant field locations, especially in forward military theatres and geographically challenging regions. Therefore, a new crystalloid low volume resuscitation solution has been developed and tested in pre-clinical hemorrhagic and trauma shock models and found to be highly effective in increasing the tolerance to the low volume state by significantly increasing microcirculatory oxygen transfer efficiency. These solutions are based on specifically sized polymers of polyethylene glycol solutes (PEG-20k) that work by osmotic and hydrophilic actions in the microcirculation. These forces non-energetically move isotonic fluid out of metabolically swollen cells into capillaries thereby reloading the exchange vessels and propelling convective oxygen transfer by decreasing the resistance to flow via their primary effects on cell and tissue swelling (tissue decompression). The result is rapid oxygen debt repayment, lactate clearance, and re-establishment of oxygen transfer under very low volume conditions. This approach is ideal for pre-hospital use because metabolic and cardiovascular tolerance to trauma increases, which can safely lengthen evacuation and transport times and ensures better outcomes when definitive resuscitation occurs at a civilian or forward military hospital. Since the active molecules in these new solutions are large polymers not unlike hydroxyethyl starch (HES) and because they produce significant water transfer and dilutional effects in blood compartments, the effects of these solutions on whole blood coagulation and platelet function are of possible concern and are as yet unknown. Therefore, the purpose of this study was to characterize the effects of LVR solutions containing PEG-20k on whole blood coagulation and platelet function using TEG analysis (this report) and on more

detailed mechanisms of coagulation and platelet function using more specific testing in a companion paper to this one.

In these experiments using ex-vivo diluted whole blood, 10% PEG-20k produces a clinically recognizable coagulopathy (i.e. requiring blood product transfusion per ACS TQIP Massive Transfusion in Trauma Best Practice Guidelines) that is statistically different from normal saline dilutional control at a 10% volume dilution, but *not* from 6% HES colloidal controls in healthy volunteers or trauma patients. The dilution factor of 10% was chosen since this is the upper limit of low volume resuscitation ranges that may be used in the field, corresponding to an approximate volume of 500 ml in an adult (with a blood volume of 5 liters). Since these studies are diluted ex-vivo, we assume the coagulation effects observed are true for patients when they are diluted at a similar 10% volume. However, in trauma patients in the field requiring low volume resuscitation, there is no way of accurately estimating if a theoretical 10% LVR dilution with crystalloid actually represents 10% or something larger or smaller. Understanding this is essential in order to extrapolate the coagulation results from this ex-vivo study that used an exact 10% dilution with the LVR solutions being tested but also showed different effects with slightly lower PEG-20k concentrations. The forces favoring a greater dilution in the trauma patient over the 10% estimated theoretical value (resulting in a lower % PEG-20k concentration) include dilution in the vascular space from subsequent movements of isotonic water from the intracellular and interstitial spaces into the capillary space, which is where coagulation and platelet function occurs. This is probably a significant dilution and can represent a doubling of the intravascular isotonic water volume that cuts hemoglobin and albumin marker concentrations in half (8, 10). An additional factor that would favor a dilution of the PEG exceeding the theoretical calculated 10% after administration to trauma patients include the capillary reflection coefficient of the solute (PEG-20k). The osmotic reflection coefficient σ_d is a measure of the % partitioning of a large solute molecule (like PEG-20k) between the capillary and the extracapillary space. A molecule that has a reflection coefficient of 1.0 demonstrates 100% reflection

by pores in the capillary so 100% stays in the capillary available for interactions with coagulation factors and platelet interactions. A coefficient of 0 indicates no reflection and the solute is equilibrated equally between the capillary and interstitial spaces, or, 50% of the material and the osmotic effect is lost. The actual σ_d of PEG-20k is 0.5 in most capillary beds (2, 10), which means that 33% of the material administered into the vascular space (10% theoretically) quickly equilibrates outside of the capillary into the interstitial space. In fact, this intermediate reflection coefficient, which is rare, was a sought-after molecular attribute for choosing an ideal impermeant solute to construct an LVR solution with maximum water transfer properties. This means that, all things being equal, administration of a 10% PEG-20k solution will result in a 6.6% solution after the solute molecules equilibrate across the capillary membrane, based on the properties of the capillary as defined by PEG-20k's unique reflection coefficient. This property, along with the large pull of water into the capillary space from the osmotic and hydrophilic forces of the PEG-20k, tends to dilute out the PEG-20k concentration in the blood and reduce the effects of the PEG polymers on interference with coagulation. Factors that tend to increase the concentration of PEG-20k from the theoretical 10% dilution after administration to trauma patients is the large hemorrhage volumes that are not taken into account when the theoretical blood volume is calculated. If 25% of the blood volume is lost in a trauma patient to hemorrhage, then administration of a 10% solution at an estimated 10% blood volume dilution will result in an underestimation of the blood volume and a more concentrated final PEG-20k concentration in the vascular space (to about 12.5%). This may tend to exacerbate any dose-dependent PEG-20k effects on the coagulation and platelet system. The final dilution of a 10% PEG-20k solution given at a theoretical 10% dilution in a trauma patient will be an algebraic sum of all of these forces acting together. Our preliminary modeling of a patient with a 40% hemorrhage volume suggest a dilution greater than 10%, which would lessen the coagulation side effects that had been documented here in ex-vivo whole patient blood at an exact 10%

dilution. This has been validated with some preliminary animal hemorrhage studies using labeled PEG-20k (data not shown).

The mechanism of the coagulopathic effects of PEG-20k LVR solutions on clotting blood cannot be determined from the TEG data because the tests are descriptive. However, certain mechanistic effects can be inferred from the individual changes in the TEG outcome variables. The TEG responses in both healthy volunteer and trauma patient blood diluted 10% with PEG-20k solutions (at 10% concentration) suggests an interference with direct platelet function (because MA is reduced) and possibly indirect effects of the platelet contribution to coagulation from thrombin generation (because K and angle are effected). Other possible effects on coagulation reactions cannot be excluded from the TEG data alone. Based on the TEG data, the polymer may create a state of functional thrombocytopenia, as opposed to a physical thrombocytopenia, because MA is lowered after dilution with PEG-20k solutions in a test tube, where the platelet counts are identical in the test PEG tubes and the dilutional control tubes, which don't exhibit changes in MA. Without further testing, it is not possible to know for sure how PEG-20k polymers affect whole blood coagulation but our preliminary hypothesis at this time is that the polymer causes a state of reversible platelet thrombocytopenia through physical platelet passivation effects, maybe by cross linking or adsorbing platelets. This would functionally remove platelets from the system and prevent their direct aggregation to form a platelet-fibrin clot and reduce the membrane and phospholipid effects of the platelet on the acceleration of coagulation reactions and the rate of fibrin formation. Further mechanistic experiments to sort out these possibilities have been conducted and the results, analysis, and conclusions are presented in the follow on companion paper.

The effects of PEG-20k on coagulation and platelet function, as assessed by TEG, were the same in blood from health volunteers and trauma patients. This is good to know since our intent is to understand how these solutions influence systems in the trauma patient. Our selection criteria for trauma patients

was strict in that we wanted severely injured patients with an Injury Severity Score (ISS) over 24, a lactate on arrival of >4.6 mM, and a hypotension characterized by a systolic blood pressure <95 mmHg. Another goal was to measure patients as soon as they entered the trauma system because they would have a greater chance of not being transfused with blood or given significant volumes of crystalloids that would further complicate an already chaotic system. It was surprising that we didn't see evidence of a trauma induced coagulopathy (TIC) in our trauma patient population, especially a temporary hypercoagulative state. We also did not follow these patients in time to document the development of a later TIC or hypocoagulative state. In any case, the effects of PEG-20k LVR solutions behaved almost identically in patients as it did in healthy volunteers.

This study compared various concentrations of PEG-20k on coagulation and platelet function using TEG compared to a dilutional saline control and a clinical (military medicine) control using a 6% solution of hydroxyethyl starch (Hextend). While both Hextend and PEG-20k solutions produced measureable and significant effects on TEG outcomes, the PEG effects were not significantly different from the Hextend effects even though the absolute changes appeared more pronounced in the 10% PEG-20k groups. The commonality between PEG-20k and HES are that they both are polymers and they both have hypocoagulative effects on whole blood TEG testing. However, these polymers are chemically different and the similarity or differences in their mechanisms of action on the coagulation and platelet activation system should be speculated with caution until more definitive mechanistic testing is performed. At this point, it appears that the qualitative effects on TEG for both polymers are very similar when comparing 6% HES with 10% PEG-20k.

In conclusion, this study clearly shows that LVR solutions used for the resuscitation of patients in severe hypovolemic shock has statistically significant but minor effects on whole blood coagulation and platelet function as determined by TEG in an ex-vivo test system. The effects are not due to volume

dilution and are similar to those seen with 6% HES. The PEG-20k effects are dose dependent and are essentially abrogated and reversed by reducing the PEG-20k concentrations from 10% to 7.5%. The exact mechanisms of the polymer effects on TEG are not known but the TEG analysis suggests that a physical platelet passivation response is occurring. The clinical effects will need to be verified in an in-vivo model.

ACKNOWLEDGEMENT

This work was supported by grants from the Department of Defense to Dr. Mangino (W81XWH-12-1-0599 and W81XWH-16-2-0040)

FIGURE LEGENDS

Figure 1: Clot initiation indexed by the **R** time on TEG in blood from healthy volunteers, trauma patients, and volunteers in a PEG-20k dose-response series. All TEG outcomes represents 25 healthy volunteers, 12 trauma patients, and 6 volunteers for dose-response studies. Whole citrate preserved blood was immediately diluted 10% with a saline vehicle (NS), 6% solution of Hextend (HS), and Polyethylene Glycol-20k (PG) at concentrations of 10%, 7.5%, or 5% and assayed by full thromboelastography (TEG) within 2-3 hours of blood draw. All values are expressed in a box and whiskers standard format where the bar in the box is the sample median value, the lower border of the box is the value demarcating the 1st and second interquartile range, the upper border of the box is the value demarcating the 3rd and 4th interquartile range, and the upper and lower whiskers are the samples highest and lowest values, respectively. The shaded box represents the known normal ranges for the TEG values. WB= whole blood (undiluted). *P<0.05.

Figure 2: Clot amplification indexed by the **K** time on TEG in blood from healthy volunteers, trauma patients, and volunteers in a PEG-20k dose-response series. All TEG outcomes represents 25 healthy volunteers, 12 trauma patients, and 6 volunteers for dose-response studies. Whole citrate preserved blood was immediately diluted 10% with a saline vehicle (NS), 6% solution of Hextend (HS), and Polyethylene Glycol-20k (PG) at concentrations of 10%, 7.5%, or 5% and assayed by full thromboelastography (TEG) within 2-3 hours of blood draw. All values are expressed in a box and whiskers standard format where the bar in the box is the sample median value, the lower border of the box is the value demarcating the 1st and second interquartile range, the upper border of the box is the value demarcating the 3rd and 4th interquartile range, and the upper and lower whiskers are the samples highest and lowest values, respectively. The shaded box represents the known normal ranges for the TEG values. WB= whole blood (undiluted). *P<0.05.

Figure 3: Clot propagation indexed by the **Angle** variable on TEG in blood from healthy volunteers, trauma patients, and volunteers in a PEG-20k dose-response series. All TEG outcomes represents 25 healthy volunteers, 12 trauma patients, and 6 volunteers for dose-response studies. Whole citrate preserved blood was immediately diluted 10% with a saline vehicle (NS), 6% solution of Hextend (HS), and Polyethylene Glycol-20k (PG) at concentrations of 10%, 7.5%, or 5% and assayed by full thromboelastography (TEG) within 2-3 hours of blood draw. All values are expressed in a box and whiskers standard format where the bar in the box is the sample median value, the lower border of the box is the value demarcating the 1st and second interquartile range, the upper border of the box is the value demarcating the 3rd and 4th interquartile range, and the upper and lower whiskers are the samples highest and lowest values, respectively. The shaded box represents the known normal ranges for the TEG values. WB= whole blood (undiluted). *P<0.05.

Figure 4: Clot strength and maximum size indexed by the **MA** on TEG in blood from healthy volunteers, trauma patients, and volunteers in a PEG-20k dose-response series. All TEG outcomes represents 25 healthy volunteers, 12 trauma patients, and 6 volunteers for dose-response studies. Whole citrate preserved blood was immediately diluted 10% with a saline vehicle (NS), 6% solution of Hextend (HS), and Polyethylene Glycol-20k (PG) at concentrations of 10%, 7.5%, or 5% and assayed by full thromboelastography (TEG) within 2-3 hours of blood draw. All values are expressed in a box and whiskers standard format where the bar in the box is the sample median value, the lower border of the box is the value demarcating the 1st and second interquartile range, the upper border of the box is the value demarcating the 3rd and 4th interquartile range, and the upper and lower whiskers are the samples highest and lowest values, respectively. The shaded box represents the known normal ranges for the TEG values. WB= whole blood (undiluted). *P<0.05.

Figure 5: Clot lysis indexed by the **LY30** on TEG in blood from healthy volunteers, trauma patients, and volunteers in a PEG-20k dose-response series. All TEG outcomes represents 25 healthy volunteers, 12 trauma patients, and 6 volunteers for dose-response studies. Whole citrate preserved blood was immediately diluted 10% with a saline vehicle (NS), 6% solution of Hextend (HS), and Polyethylene Glycol-20k (PG) at concentrations of 10%, 7.5%, or 5% and assayed by full thromboelastography (TEG) within 2-3 hours of blood draw. All values are expressed in a box and whiskers standard format where the bar in the box is the sample median value, the lower border of the box is the value demarcating the 1st and second interquartile range, the upper border of the box is the value demarcating the 3rd and 4th interquartile range, and the upper and lower whiskers are the samples highest and lowest values, respectively. The shaded box represents the known normal ranges for the TEG values. WB= whole blood (undiluted)

Figure 6: Coagulation Index as measured by the **CI** on TEG in blood from healthy volunteers, trauma patients, and volunteers in a PEG-20k dose-response series. The CI is a mathematical compilation of other TEG outcome variables (R, K, Angle, and MA). All TEG outcomes represents 25 healthy volunteers, 12 trauma patients, and 6 volunteers for dose-response studies. Whole citrate preserved blood was immediately diluted 10% with a saline vehicle (NS), 6% solution of Hextend (HS), and Polyethylene Glycol-20k (PG) at concentrations of 10%, 7.5%, or 5% and assayed by full thromboelastography (TEG) within 2-3 hours of blood draw. All values are expressed in a box and whiskers standard format where the bar in the box is the sample median value, the lower border of the box is the value demarcating the 1st and second interquartile range, the upper border of the box is the value demarcating the 3rd and 4th interquartile range, and the upper and lower whiskers are the samples highest and lowest values, respectively. The shaded box represents the known normal ranges for the TEG values. WB= whole blood (undiluted). Tracings on the left are representative TEG tracings obtained from all three LVR solutions used in this study (NS, PEG-20k, and HES). *P<0.05

Figure 7: Acute resuscitation outcomes in a study in rats subjected to severe hemorrhagic shock and low volume resuscitation with NS (saline) controls, or 7.5% and 10% PEG-20k. All LVR solutions were given at a volume equal to 10% of the estimated blood volume of the animals. The low volume resuscitation times (LVR) were measured and shown in panel A, which is an index of tolerance to the low volume state (see methods for details), the end or terminal lactate values are shown in panel B, which are the values at the end of the LVR period (or 240 minutes in the PEG groups), and the terminal mean arterial blood pressure measured at the end of the LVR period in panel C. Values are mean +/- standard deviation, n= 12 for NS and 10% PEG-20k and n=4 for 7.5% PEG-20k. *P<0.05 relative to both PEG groups.

Fig 1

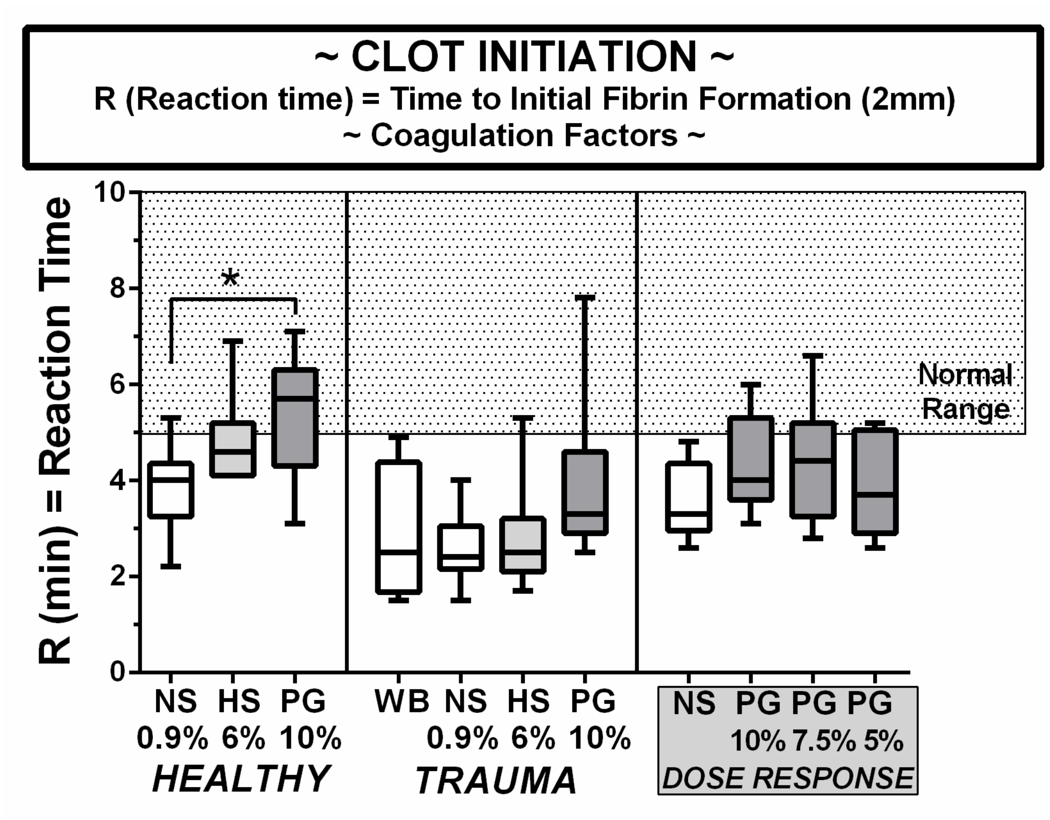

~ CLOT INITIATION ~
R (Reaction time) = Time to Initial Fibrin Formation (2mm)
~ Coagulation Factors ~
R (min) = Reaction Time
10
8
6
4
2
0
*
Normal Range
NS 0.9%
HS 6%
PG 10%
HEALTHY
WB
NS 0.9%
HS 6%
PG 10%
TRAUMA
NS
PG 10%
PG 7.5%
PG 5%
DOSE RESPONSE

Fig 2

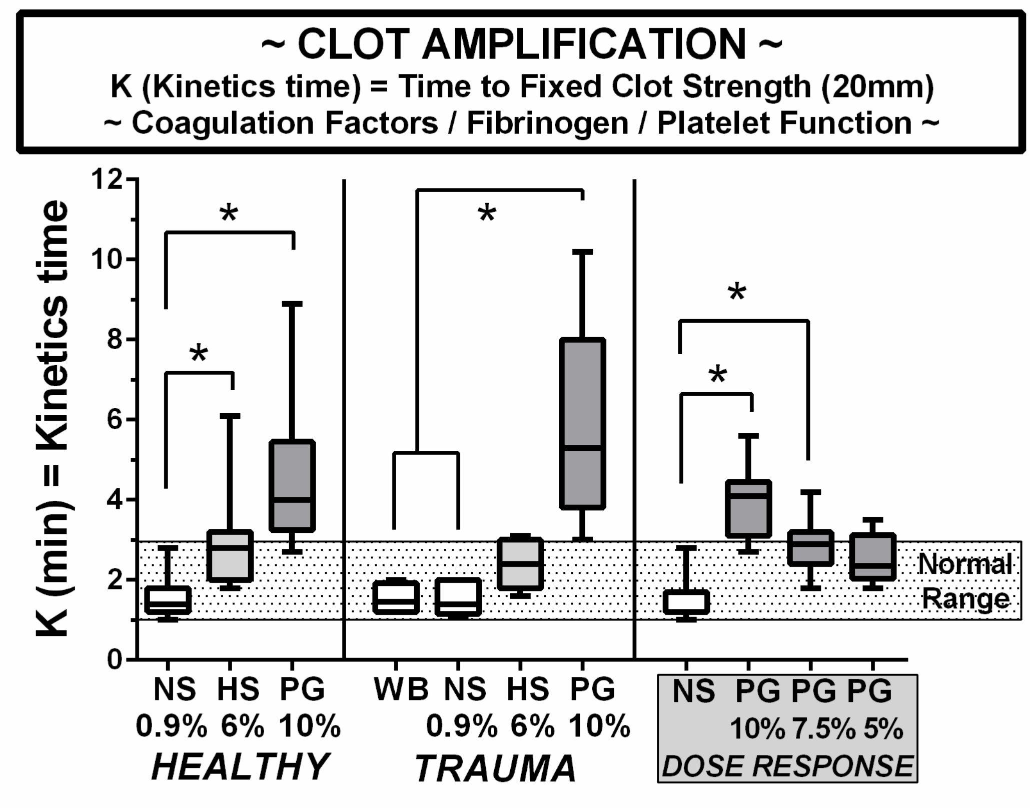

~ CLOT AMPLIFICATION ~
K (Kinetics time) = Time to Fixed Clot Strength (20mm)
~ Coagulation Factors / Fibrinogen / Platelet Function ~
K (min) = Kinetics time
12
10
8
6
4
2
0
*
*
*
*
*
Normal
Range
NS 0.9%
HS 6%
PG 10%
HEALTHY
WB
NS 0.9%
HS 6%
PG 10%
TRAUMA
NS
PG 10%
PG 7.5%
PG 5%
DOSE RESPONSE

Fig 3

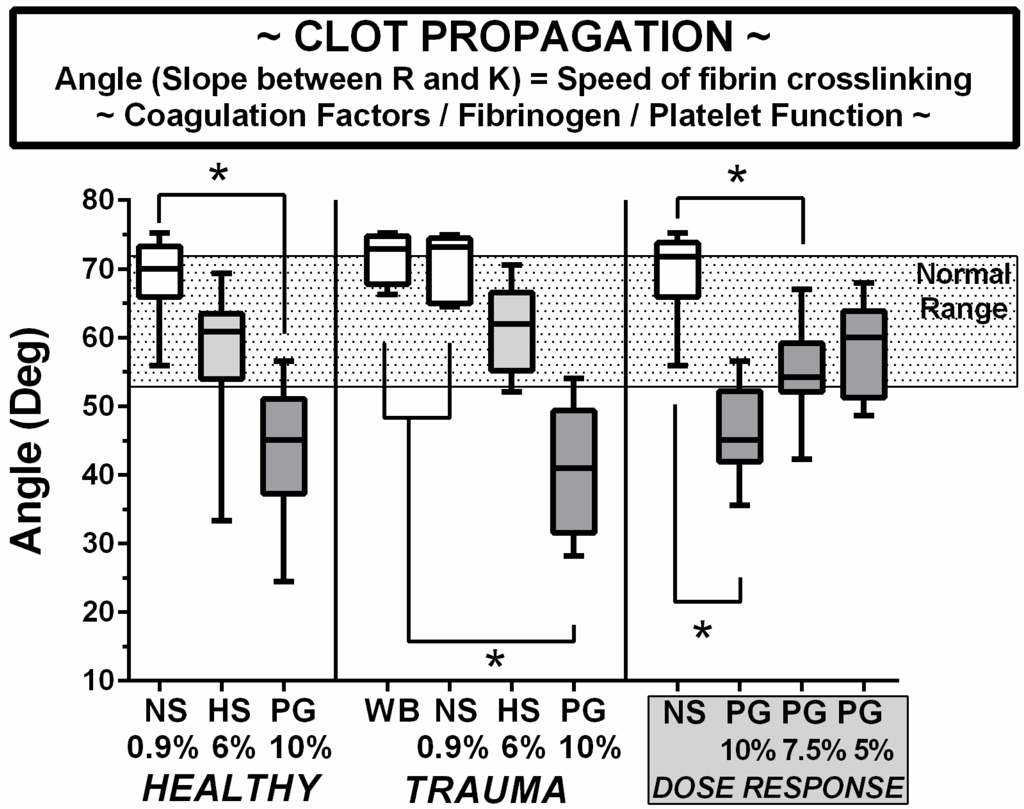
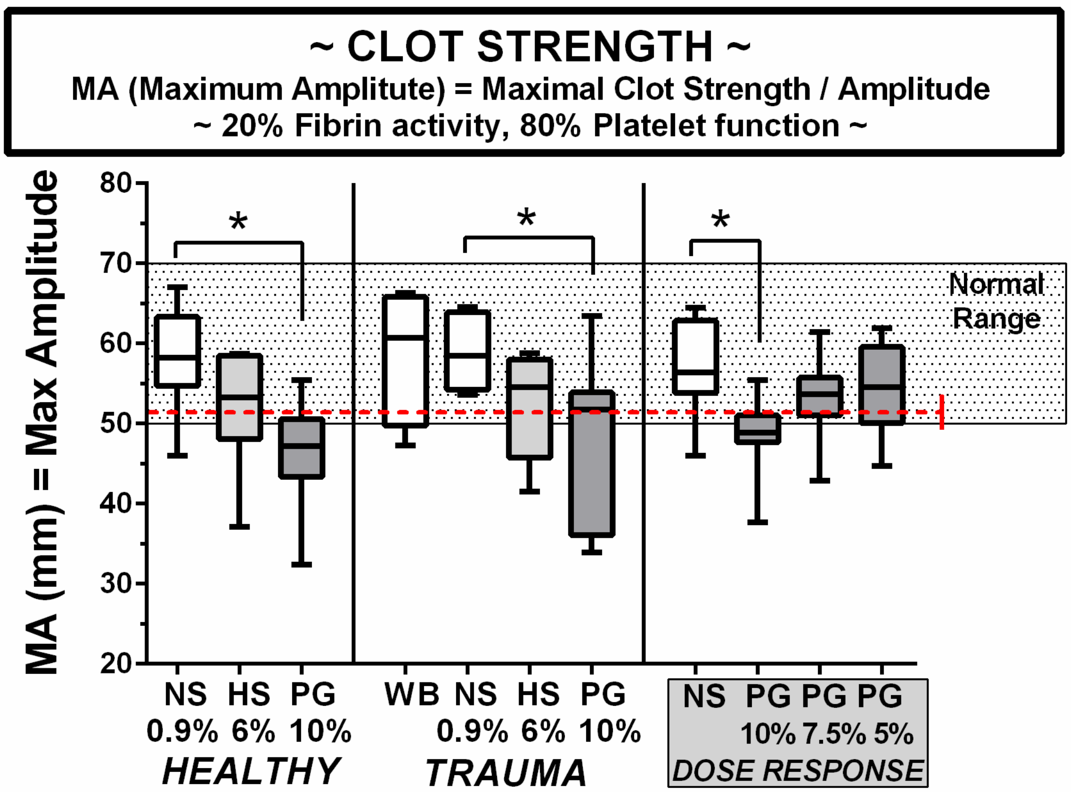

~ CLOT PROPAGATION ~
Angle (Slope between R and K) = Speed of fibrin crosslinking
~ Coagulation Factors / Fibrinogen / Platelet Function ~
Angle (Deg)
80
70
60
50
40
30
20
10
Normal Range
*
*
*
*
NS 0.9%
HS 6%
PG 10%
HEALTHY
WB
NS 0.9%
HS 6%
PG 10%
TRAUMA
NS
PG 10%
PG 7.5%
PG 5%
DOSE RESPONSE

Fig 4



~ CLOT STRENGTH ~
MA (Maximum Amplitute) = Maximal Clot Strength / Amplitude
~ 20% Fibrin activity, 80% Platelet function ~
MA (mm) = Max Amplitude
80
70
60
50
40
30
20
*
*
*
Normal Range
NS 0.9%
HS 6%
PG 10%
HEALTHY
WB
NS 0.9%
HS 6%
PG 10%
TRAUMA
NS
PG 10%
PG 7.5%
PG 5%
DOSE RESPONSE

Fig 5

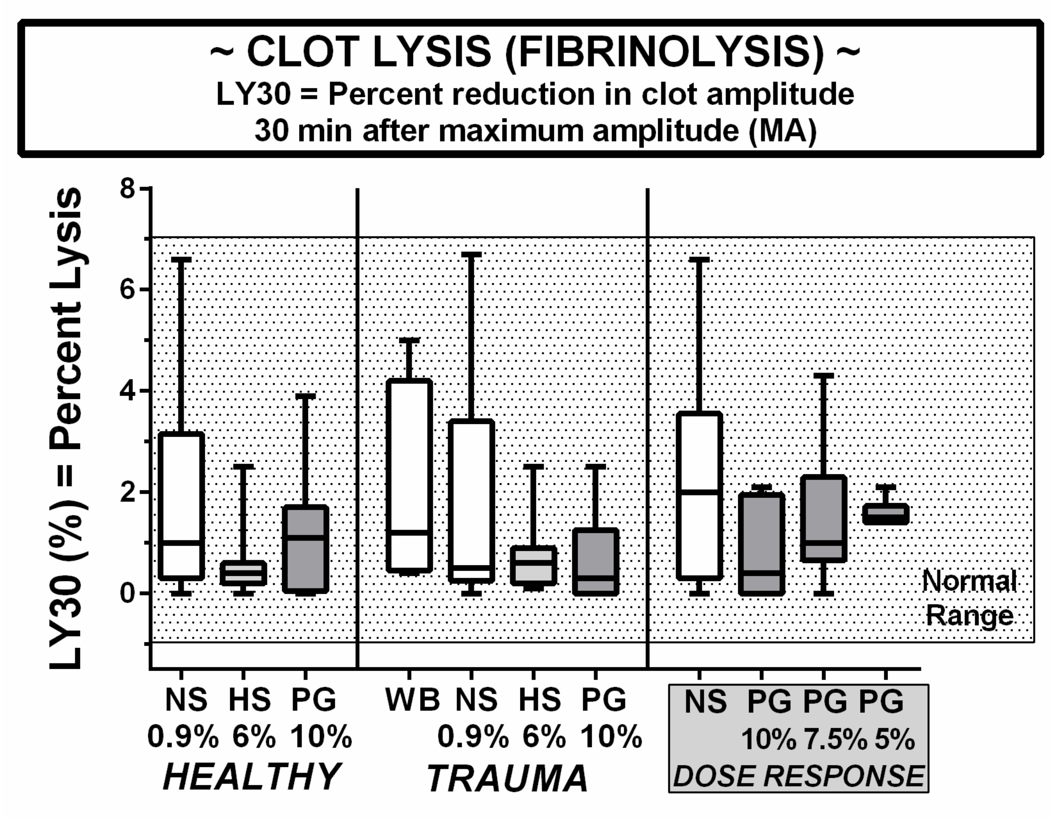
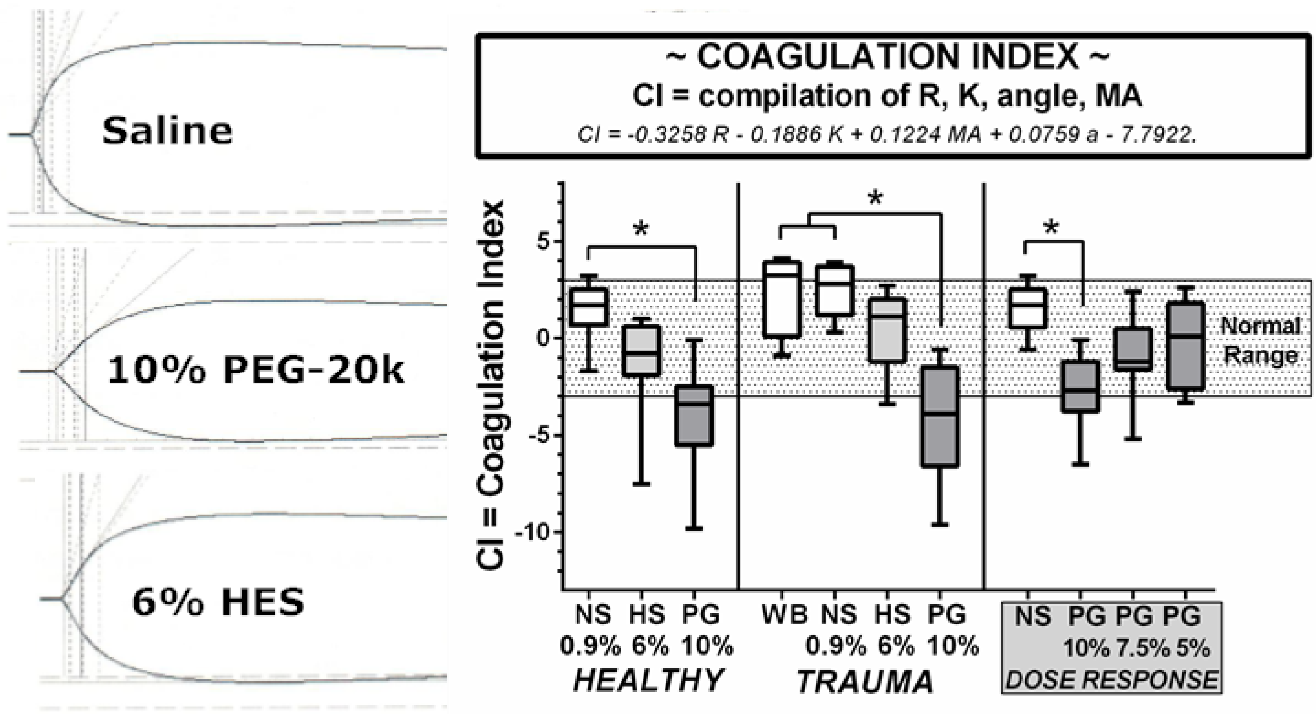
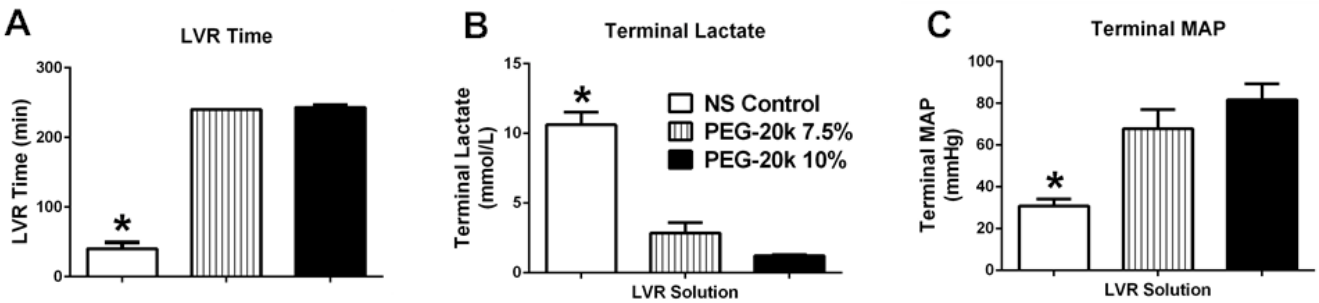

~ CLOT LYSIS (FIBRINOLYSIS) ~
LY30 = Percent reduction in clot amplitude
30 min after maximum amplitude (MA)
LY30 (%) = Percent Lysis
8
6
4
2
0
Normal
Range
NS 0.9%
HS 6%
PG 10%
HEALTHY
WB
NS 0.9%
HS 6%
PG 10%
TRAUMA
NS
PG 10%
PG 7.5%
PG 5%
DOSE RESPONSE

Fig 6



Saline
10% PEG-20k
6% HES
~ COAGULATION INDEX ~
CI = compilation of R, K, angle, MA
CI = -0.3258 R - 0.1886 K + 0.1224 MA + 0.0759 a - 7.7922.
CI = Coagulation Index
5
0
-5
-10
Normal Range
NS 0.9%
HS 6%
PG 10%
HEALTHY
WB
NS 0.9%
HS 6%
PG 10%
TRAUMA
NS
PG 10%
PG 7.5%
PG 5%
DOSE RESPONSE

Fig 7


A
LVR Time
LVR Time (min)
300
200
100
0
*
B
Terminal Lactate
Terminal Lactate (mmol/L)
15
10
5
0
*
NS Control
PEG-20k 7.5%
PEG-20k 10%
LVR Solution
C
Terminal MAP
Terminal MAP (mmHg)
100
80
60
40
20
0
*
LVR Solution

References

1. van Lambalgen AA, van den Bos GC, Thijs LG. Whole body plasma extravasation in saline and Haemaccel loaded rats: effects of endotoxemia. IntJMicrocircClinExp. 1990;9(3):303-18.

2. Parrish D, Plant V, Lindell SL, Limkemann A, Reichstetter H, Aboutanos M, et al. New low-volume resuscitation solutions containing PEG-20k. JTrauma Acute Care Surg. 2015;79(1):22-9.

3. Stein DM, Scalea TM. Capillary leak syndrome in trauma: what is it and what are the consequences? AdvSurg. 2012;46:237-53.

4. Duchesne JC, McSwain NE, Jr., Cotton BA, Hunt JP, Dellavolpe J, Lafaro K, et al. Damage control resuscitation: the new face of damage control. JTrauma. 2010;69(4):976-90.

5. Riha GM, Kunio NR, Van PY, Hamilton GJ, Anderson R, Differding JA, et al. Hextend and 7.5% hypertonic saline with Dextran are equivalent to Lactated Ringer's in a swine model of initial resuscitation of uncontrolled hemorrhagic shock. JTrauma. 2011;71(6):1755-60.

6. Riha GM, Kunio NR, Van PY, Kremenevskiy I, Anderson R, Hamilton GJ, et al. Uncontrolled hemorrhagic shock results in a hypercoagulable state modulated by initial fluid resuscitation regimens. JTrauma AcuteCare Surg. 2013;75(1):129-34.

7. Cotton BA, Guy JS, Morris JA, Jr., Abumrad NN. The cellular, metabolic, and systemic consequences of aggressive fluid resuscitation strategies. Shock. 2006;26(2):115-21.

8. Plant V, Limkemann A, Liebrecht L, Blocher C, Ferrada P, Aboutanos M, et al. Low-volume resuscitation using polyethylene glycol-20k in a preclinical porcine model of hemorrhagic shock. The journal of trauma and acute care surgery. 2016;81(6):1056-62.

9. Parrish D, Lindell SL, Reichstetter H, Aboutanos M, Mangino MJ. Cell Impermeant-based Low-volume Resuscitation in Hemorrhagic Shock: A Biological Basis for Injury Involving Cell Swelling. Ann Surg. 2016;263(3):565-72.

10. Plant V, Parrish D, Limkemann A, Ferrada P, Aboutanos M, Mangino MJ. Low Volume Resuscitation for Hemorrhagic Shock: Understanding the Mechanism of PEG-20k. J Pharmacol Exp Ther. 2017.

11. Mangino MJ, Liebrecht LK, Plant V, Limkemann A. Crystalloid and Colloid Resuscitation: Hypertonic Saline, Starches, Polymers, and Gelatins. In: Pascual JL, Cannon JW, editors. Hemorrhagic Shock: Recognition, Pathophysiology, and Management. Hauppauge, NY: Nova Publishers; 2017.

12. Arora TK, Malhotra AK, Ivatury R, Mangino MJ. L-arginine infusion during resuscitation for hemorrhagic shock: impact and mechanism. JTrauma AcuteCare Surg. 2012;72(2):397-402.

HESPAN reference:

https://www.fda.gov/downloads/BiologicsBloodVaccines/BloodBloodProducts/ApprovedProducts/NewDrugApplicationsNDAs/UCM376787.pdf

Mechanisms of Novel Polyethylene Glycol Based Low Volume Resuscitation Solutions on Coagulation and Platelet Function

Loren K. Liebrecht, M.D.[1], Jason Newton, Ph.D.[2] Erika J. Martin, M.S.[5], Nina Wickramaratne, M.D.[1], Sudha Jayaraman, M.D.[1], Jinfeng Han, B.S.N. [1], Michel Aboutanos, M.D.[1], Donald F. Brophy, Pharm.D.[5], and Martin J. Mangino, Ph.D.[1,3,4]

[1]Department of Surgery, Division of Acute Care Surgery, [2]Department of Biochemistry and Molecular Biology, [3]Department of Physiology and Biophysics, [4]Department of Emergency Medicine, School of Medicine, and [5]Department of Pharmacotherapy and Outcomes Science, School of Pharmacy, Virginia Commonwealth University, Richmond, VA 23298

Corresponding Author
Martin J. Mangino
Department of Surgery
Virginia Commonwealth University
MCV Campus
1101 E. Marshall St.
Richmond, VA 23298
804-628-3226
804-828-8300 (FAX)
mjmangino@vcu.edu

Support: This work was supported by the Department of Defense W81XWH-16-2-0040 and W81XWH-12-1-0599 to Dr. Mangino

Running Head: Sites of polymer interference in clot formation.

ABSTRACT

Novel crystalloid solutions containing polyethylene glycol polymers (PEG-20k) produce dramatic resuscitation effects but dose dependently can produce a mild hypocoagulative state assessed on Thromboelastography (TEG). The objective of this study was to examine possible mechanisms of this effect. Whole citrated blood from healthy volunteers was diluted ex-vivo 10% with crystalloids and tested for coagulation and platelet function. Fibrinogen and von Willebrand factor (vWF) concentrations were not affected by PEG-20k. The Prothrombin Time (PT) and activated Partial Thromboplastin Time (aPTT) were also not affected by PEG-20k dilutions, relative to saline dilutional controls. However, the silica activator used for aPTT testing gave a false positive state of intrinsic pathway inactivation. Thrombin activity assessed using the Calibrated Automated Thrombogram (CAT) was mildly suppressed with PEG-20k LVR solutions (ETP- 24%; Peak- 27%; TTP- 20%). Platelet mapping studies demonstrated significantly greater % inhibition of both ADP and arachidonic acid-induced platelet aggregation with PEG-20k, compared to dilutional controls, but direct ADP-activated gpIIa/IIIb (PAC1) and P-selectin (CD62P) binding site expression was not altered by PEG-20k using fluorescence cytometry. Erythrocyte Sedimentation Rates (ESR) were dramatically accelerated (150x) after dilution with 10% PEG-20k, compared to the saline controls, and this was competitively blocked by smaller PEG polymers between 1-8 kDa, suggesting nonspecific cell binding effects of PEG-20k. In conclusion, PEG-20k creates a mild hypocoagulative state in whole blood at concentrations ≥10%, which are mainly due to platelet effects and not coagulation. This may be due to a functional thrombocytopenia induced by nonspecific platelet surface passivation by the PEG polymers.

INTRODUCTION

Trauma is the number one cause of death for people under 44 years of age in the US and the third leading cause of death overall for all age groups. Trauma accounts for about 30% of all life-years lost in the US, compared to cancer (16%), heart disease (12%), and HIV (2%) (1). For all traumatic injuries, hemorrhagic shock is responsible for over 35% of pre-hospital deaths and over 40% of all deaths within the first 24 hours. This is second only to deaths induced by severe CNS injury (2). Hemorrhagic hypotension exposes the patient to immediate complications of life-threatening infections, coagulopathies, and multiple organ failure (3, 4). Crystalloid-based I.V. solutions are available for pre-hospital use because they can be safely transported and stored but they are generally limited in their effectiveness. Only a fraction of infused crystalloid volume stays in the intravascular space and the use of low volume crystalloids has minimal effects on pressure and perfusion (5, 6). The movement of crystalloid fluid from capillary to interstitium is compounded by the increase in capillary permeability from trauma-related inflammation and trauma-induced capillary leak syndrome (TICS) (7). Furthermore, crystalloid resuscitation exacerbates TICS, acidosis, hypothermia, and coagulopathy (7, 8). Other resuscitation solutions such as hypertonic saline or starch have had disappointing results (9, 10) including concerns and risks associated with their use (8, 11). There remains a need for a better crystalloid fluid that can be given at a low volume to resuscitate patients in severe hemorrhagic shock awaiting definitive treatment, especially for the prehospital setting.

Recently, polyethylene glycol polymers of specific molecular weight ranges have been used in crystalloid solutions to act as highly effective LVR solutions (6, 12-14). These polymers non-energetically move isotonic fluid from intracellular and interstitial spaces into the capillary space by simple osmotic actions in response to metabolic cell swelling that occurs in shocked and ischemic

tissues. As water flow moves from the interstitial spaces to the capillaries, the capillary exchange in the tissues dramatically improves under very low volume conditions because the microcirculation is decompressed while the capillary spaces are re-loaded with volume and pressure for driving flow (14). This causes rapid clearance of lactate, increased blood pressure, and tolerance to the low volume state (12). While these polymers work several fold better than hydroxyethyl starch based polymers, implying different mechanisms of action, interference with blood clotting and coagulation may be shared by both types of polymers. Intravenous admiration of Hextend and Hespan are complicated by both renal toxicity and coagulopathies (15), which in trauma settings are a concern. In the companion paper to this report, we describe detailed evidence of a mild hypocoagulative state induced by 10% dilutions of whole blood from volunteers and trauma patient blood with 10% PEG-20k I.V. solutions. The TEG based data suggest PEG-20k has effects on platelet components of clot formation (MA) but changes in clot propagation on TEG can't rule out effects on the coagulation system too. Therefore, this study was conducted to drill down to the more detailed mechanisms of the dose dependent hypo-coagulation seen with solutions of PEG-20k low volume resuscitation solutions. To that end, we studied coagulation and platelet function outcomes in fresh whole blood obtained from healthy volunteers diluted 10% with IV resuscitation solutions including PEG-20k.

METHODS

Volunteer Blood: Whole blood (15-ml) was drawn into citrated vacutainer collection tubes from 52 healthy volunteers (18-50 years of age) of both sexes that were free of all medications and tobacco. The blood was diluted 10% in the lab with solutions of 10%, 7.5%, and 5% PEG-20k in saline (0.9% NaCl), 6% Hextend, or a 0.9% NaCl solution that served always as a paired dilutional control for all test solutions. Immediately after the dilutions, the samples were taken to the coagulation lab for analysis. One mL of citrated whole blood aliquots was used for TEG analysis using Kaolin as activator, and the remaining citrated plasma was centrifuged at 180 x g for 10 minutes to obtain platelet rich plasma (PRP). Platelet poor plasma (PPP) was obtained by double centrifugation of the remaining plasma at 2000 x g for 10 min at room temperature. PRP was then diluted with autologous PPP to yield a final platelet count of 150 $x10^9$/L for platelet-dependent thrombin generation assays. The remaining PPP was used for the analysis of platelet-independent thrombin generation, PT, aPTT, Fibrinogen, and vWF assays. The time between blood draw and analysis was typically under 2 hours. All volunteers donated blood under a VCU approved IRB protocol.

Fibrinogen , PT, aPTT, vWF: Fibrinogen, PT, aPTT, and von Willebrand factor antigen (vWF) were measured in plasma using standard assays (STA fibrinogen clotting activity assay, PT-Neoplastin CI, PTT- Automate, PTT CK Prest, and Liatest vWF assays, respectively) on the STA Compact analyzer (Diagnostica Stago, Parsippany, NJ, USA) according to manufacturer’s instructions.

CAT Assay: The kinetics of thrombin generation was assessed in PRP and in PPP according to methods previously described by Hemker, et al (16). Briefly, 20 µl of trigger reagent (1pM Tissue

Factor), and 80 μl PPP were manually pipetted in triplicate into 96-well microtiter plates (Immulon 2HB plate; Diagnostica Stago, Parsippany, NJ, USA). The plate was placed in the fluorometer for a 10 minute 37°C incubation (Fluoroskan AscentTM; Thermolab Systems OY, Helsinki, Finland). The device was equipped with a 390/460 filter set. Twenty μl of starting reagent containing the fluorogenic substrate Z-GGR-AMC (2.5 mM) and CaCl2 (100 mM) were automatically dispensed into each well immediately before measurement initiation. Thrombin generation curves were calculated using the calibrated automated thrombogram (Thrombinoscope BV, Masstricht, The Netherlands) software version: V5.0.0.742. Thrombogram parameters, lag time, peak thrombin and endogenous thrombin potential (ETP), which reflected the maximum amount of thrombin that a sample could potentially generate, were reported.

Platelet Mapping Study: Thromboelastography and Platelet Mapping: Clot formation was determined with a Thrombelastography Analyzer (TEG 5000; Haemonetics Corp, Braintree, Mass) using the intrinsic pathway activator kaolin (Haemonetics Corp) and recalcification to 10 mmol/L final calcium concentration. The TEG 5000 reports time to onset of clot formation (R), which positively correlates with thrombin generation; the time to reach a predetermined level of clot stiffness (K) and the clotting angle (Angle), which correlates with fibrin polymerization; the maximal amplitude (MA) or stiffness, representing clot strength. Platelet mapping was done using the TEG-5000 instrument and a platelet mapping kit (Haemonetics) that tests the platelet component to clot formation (MA) on TEG (17). Heparinzed blood treated with reptilase and factor VIII is used to isolate only the fibrin component of clot formation and the platelet specific component specific for the ADP and thromboxane receptor pathways are determined by activation with ADP and arachidonic acid (AA), respectively, on the heparinized blood samples.

Fluorescence Cytometry: Flow cytometric analysis was performed using citrated whole blood according to current standards from the European Working Group on Cell Analysis (18). CD41a conjugated with PE-Cy5 (Mouse Anti-Human, BD Pharmingen, Franklin Lakes, NJ, USA), PAC-1 conjugated with FITC (BD Biosciences, San Jose, CA, USA), and CD62p conjugated with PE (Mouse Anti-Human, BD Pharmingen) were used to identify platelets and to identify their activation status. Corresponding isotypic-matched monoclonal antibodies PE-Mouse IgG1-K isotype, FITC-Mouse IgM-K isotype and PE-Cy5-Mouse IgG1-K isotype (BD Pharmingen) were used as negative controls. A portion of the whole blood specimens was treated with 0.005 mL of adenosine diphosphate for platelet activation. Samples were analyzed under the following conditions: Fluidics: medium; Forward scatter threshold: 30,000; and 20,000 events were collected in a preset platelet gate using standard methods including CD41a as a global platelet marker. Results are expressed in mean fluorescence intensity units for CD41 and in percentages for other markers of activation. Flow cytometry was used to measure platelet activation via the glycoprotein P-selectin, because it is rapidly translocated to the platelet surface on stimulation. The P-selectin content on the platelet surface is detected with the CD62-P mAb. Also measured is the gp IIb/IIIa surface integrin transition to its high-affinity state by using the mAb against high-affinity glycoprotein IIb/IIIa platelet surface integrin (PAC-1) conjugated with fluorescein isothiocyanate (BD Bioscienc-es, San Jose, CA, USA). These assays provide an estimate of the platelet activation state.

Platelet Counting: Platelet counts were performed with an automated cell counter (ABX Micros 60, Horiba Medical, Irvine, CA.

ESR Study: The erythrocyte sedimentation rate is measured in diluted citrated whole blood using the Sediplast Westergren ESR system tests (Polymedco, Inc., Cortiandt Manor, NY). About 1 ml of whole blood is drawn up into a 10 cm Westergren ESR tube, which is held in the vertical position for 75 minutes. The rate of red blood cell sedimentation is measured as the migration (in mm) of the red cell column down the tube under the force of gravity. Blood samples diluted with 10% volume of saline (volume control) are compared to 10% dilution with PEG-20k solutions and PEG-20k solutions with other test compounds.

Statistical Data Analysis: All statistical analysis was performing using GraphPad Prism version 6.07 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Data groups were analyzed for outliers using the nonlinear regression ROUT method with Q=1%, the maximum desired false discovery rate. Normality of Gaussian distribution was then assessed using the D'Agnostino-Pearson ombinus K2 method. Most data were then analyzed by the non-parametric ANOVA Kruskal-Wallis test with the Mann Whiteney U test for multiple comparisons of means. Most data are expressed as the median with the 2nd and 3rd interquartile ranges and the upper and lower extremes (box and whiskers). ESR competitive inhibitor data were analyzed by nonlinear regression analysis. A P value of 0.05 was set to be the statistical cut off.

RESULTS

Plasma concentrations of fibrinogen and von Willebrand factor (vWF) were measured in PPP obtained from healthy volunteers that had been diluted 10% with either the experimental LVR solution PEG-20k (10% w:v) or a saline dilution control. These data are shown in **Figure 1** and clearly indicate that there is no difference in fibrinogen concentrations (panel A) due to dilution with PEG-20k LVR solutions. A small but statistically significant decrease in vWF (panel B) was seen for PEG-20k but the levels in this group were still within the normal range.

Coagulation of PPP was next determined in volunteer PPP diluted with either saline or PEG-20k at 10%, which is the theoretical dilution that occurs when the solutions are administered to shocked patients. **Figure 2** shows that PT times (panel A), which measure the extrinsic coagulation pathway, are both normal and not different between the two dilution solutions. The intrinsic coagulation pathway that is measured by the aPTT times (panel B) was also within normal limits for both groups and not different between the two groups when the activator contained a combination of kaolin and rabbit brain phospholipids. However, when the activator for this test contained micronized silica instead of kaolin (and rabbit brain phospholipids), there was a very significant increase in the aPTT time in the PEG-20k diluted plasma samples, relative to the saline controls, suggesting that silica interferes with initiation of the intrinsic pathways cascade. So while PEG-20k did not have any significant effects on coagulation, it dramatically prolongs the aPTT times when micronized silica is used as an activator. This is an artifact that should be avoided when testing PEG-20k diluted blood.

The thrombin activity of plasma diluted with either the saline control vehicle or PEG-20k was also determined by the Calibrated Automated Thrombogram (CAT) test. These results are shown in **figure**

3. A representative Thrombogram is shown in panel A. In this test, thrombin generation by activated plasma is detected by the ability of the formed and activate thrombin to enzymatically cleave a fluorescence-labelled pseudosubstrate, which causes fluorescence activity in the sample to rise proportional to the thrombin activity over time. The device corrects for the calibrator effects producing the final Thrombogram or thrombin production curve. The specific attributes of the Thrombogram are shown in panels B-F and describe different ways of expressing thrombin generation in the samples and between the two groups of treatments. In panel B, the ETP parameter of the thrombogram is shown, which represents the area under the thrombin generation curve that is proportional to the amount of thrombin generated during the test. Panel C shows the Peak value of the thrombogram, representing the highest amount of thrombin generation. Panel D shows the velocity index or rate of thrombin generation at its start and panels E and F show the lag phase of the start of thrombin generation from the time of activation and the time to reach peak thrombin activity, respectively. In all parameters of the CAT curve, the values for the PEG-20k group were significantly different from the corresponding values from the same blood diluted with the same volume of saline. The differences in these groups were about 25% and indicate a slight but significant reduction in thrombin generation in plasma diluted 10% with PEG-20k solutions compared to the corresponding paired 10% dilutional saline control.

The platelet contribution to clot formation under specific platelet stimulation conditions with either ADP or arachidonic acid (AA) was conducted in a platelet mapping TEG study using blood diluted with saline (control) or PEG-20k. These results are shown in **Figure 4**. Platelet activation with both ADP and arachidonic acid induced a normal clot formation response on TEG as shown by the high aggregation response (Panel B) and low inhibition response (Panel A). However, dilution with 10%

PEG-20k caused a significant inhibition of the ADP and AA response (Panel A) and a significant decrease in the ADP and AA-induced aggregation response (Panel B), relative to the saline control.

ADP activation of platelets in PRP induces a rapid expression of IIb / IIA protein complexes and P-Selectin that are detected by specific binding of antibodies to both (PAC1 and anti-CD62P, respectively). These data are shown in **Figure 5** for both saline and PEG-20k diluted PRP samples. While there were significant increases in both PAC1 (88.5%) and CD62P (59.7%) antibody binding to ADP-activated platelets, compared to the non-activated state with saline dilution, the effect was not different when PEG-20k was used as the diluent (87.4% increase for PAC1 and 62.5% increase for CD62P).

Finally, in an attempt to understand the cell binding effects of PEG-20k in blood, we used erythrocyte sedimentation as a model for what may be happening in the platelet fraction with PEG-20k. Erythrocyte Sedimentation Rates (ESR) are shown in **Figure 6** for both volunteer blood diluted with saline and PEG-20k solutions. The ESR was significantly and dose-dependently increased with 7.5% and 10% PEG-20k solutions diluted 10% with whole blood (Panel A). Addition of 10% PEG-20k (weight to volume) induced a 150 fold increase in the rate of erythrocyte sedimentation, compared to the 0% PEG-20k saline control at the same 10% volume dilution. In another study, the ESR sedimentation effect of 10% PEG-20k could be competitively inhibited by the addition of shorter chain PEG polymers of 1k, 4k, and 8k (8k shown in panel B).

DISCUSSION

A new low volume resuscitation crystalloid solution has recently been developed that induces profound tolerance to the low volume state when compared to other commonly used solutions. In preliminary testing using thromboelastography, it was determined that these solutions, which contain 10% polyethylene glycol – 20,000 (PEG-20k), produced a dose dependent hypocoagulative state. The TEG data reported in the previous companion paper indicated a significant decrease in MA and changes in angle (α) and k with PEG-20k solutions. Since MA represent clot firmness associated with platelets (80%) and fibrin (20), it was concluded that PEG-20k effects on coagulation may be mainly due to interference with platelet function over fibrin formation. However, PEG-20k-induced changes in coagulation could not be ruled out since indicators of clot propagation (α and k) were also affected. These changes have been associated with the speed of fibrin formation due to low factor activity, factor deficiency, low fibrinogen, or thrombocytopenia / thrombocytopathy. Therefore, to dissect out effects of PEG on coagulation or platelet function, we conducted more specific testing on diluted volunteer whole blood to compliment the TEG data (companion paper).

The levels of fibrinogen and vWF were not different with PEG-20k diluted blood compared to the saline controls and their concentrations were both within the normal ranges. Therefore, the low angle and k seen on TEG were not attributable to the lack of fibrinogen available for clot formation. Intuitively, the fibrinogen concentrations were identical in both groups at the start because the blood was split from the same donor for the additions of either saline or the PEG-20k solution. These data, however, suggest that the chemical amount of fibrinogen and vWF were not different and probably did not cause the slower propagation phase of clot formation with PEG-20k solutions. This may rule out a potential interference or chemical interaction of PEG polymers with fibrinogen or vWF.

Further evidence that the coagulation components of blood clotting are not involved in the hypocoagulative state associated with PEG-20k dilution was the observation that the PT and aPTT times were not different between the two groups of diluents. This is strong evidence that neither defects in the intrinsic pathway (aPTT) nor the extrinsic pathway (PT) rates of coagulation were mechanistically involved in the previously observed changes in TEG (α, k, and MA) with PEG-20k. Although PEG-20k had no effects on aPTT, we observed that activator choices for this test can give a false negative effect. Specifically, the use of an activator containing micronized silica particles to start the intrinsic pathway cascade caused very high clotting times in the presence of PEG-20k. Individual factor activity within this pathway (Factor VIII and Factor XII) were also extremely high (data not shown) but these times completely normalized when kaolin was substituted for silica as the surface activation component. The mechanisms for this silica effect are not known but may be due to a preferential adherence of PEG polymers to the silica, thereby preventing its activation of factors in the intrinsic pathway. Whatever the mechanism, it is important that any future clinical aPTT testing in patients that were given PEG-20k active solutions be tested using kaolin-based activators and not micronized silica activators. Otherwise, this will result in false coagulation results and inappropriate clinical treatment of a coagulopathy that in fact does not exist.

Thrombin generation is an important component of blood clotting and should be evaluated when a coagulopathy is identified since it represents the final common effector pathway. Furthermore, platelet dependent or independent thrombin activity may be a more important measure of coagulation than PT and aPTT times (16). Thrombin generation, as indexed by the CAT assay, indicated a slight but significant decrease in multiple measures of thrombin generation in the CAT assay system in

blood diluted with PEG-20k solutions, compared to the saline controls. These included changes in CAT Peak, CAT lag time, CAT ETP (area under the thrombin curve) and CAT Time to Peak. Although thrombin generation changes were statistically significant, they represented only a 20-30% change and may not reflect the true change in thrombin generation because the test was done in plasma only where the platelet thrombin generation component was presumably missed. The platelet component to thrombin generation, and presumably any effects due to PEG-20k interactions with platelets, may be significant. It is unclear if a 20-30% change in CAT in plasma by PEG-20k can explain alone the effects seen on TEG-MA and TEG-k (previous paper). Therefore, it may make sense to also capture thrombin generation changes by the platelet components of activated whole blood, which were not done in this study.

While the coagulation component of blood clot formation may not be a significant target of PEG-20k in clot formation at high doses, the platelet activation components may be a more significant causative target of PEG-20k effects. Platelet receptor expression (PAC1 and CD62P) after ADP activation was not altered by PEG-20k, but the functional effects of ADP activation on the platelet component of clot formation was, as seen with platelet mapping using stimulation with both ADP and arachidonic acid. This indicates that interference by PEG-20k in platelet clot formation may be downstream from IIb/IIIa receptor expression after activation. It is tempting to suggest, based on the available evidence to date, that PEG-20k may interfere with IIa/IIIb binding to fibrinogen, thereby interfering with platelet aggregation per se and the amplification of downstream receptor signaling by epinephrine, ADP, PAF, collagen, and thromboxanes on platelet aggregation. This is supported by the data showing the MA on platelet mapping and in regular TEG to be reduced with PEG-20k. Furthermore, the lower k and angle values seen with PEG-20k solutions (previous paper), which

mimic a functional state of hypofibrinogenemia in the presence of normal fibrinogen levels, may be due to blocking of the IIb/IIIa receptor and inhibition of fibrinogen binding and platelet aggregation. Therefore, PEG-20k may induce a state of chemical thrombasthenia at higher concentrations while not significantly affecting the chemical coagulation cascades. This is further supported, albeit indirectly, by data demonstrating robust effects of PEG-20k solutions on red blood cell sedimentation rates, which are competitively inhibited by smaller PEG polymers. These data suggest that PEG-20k polymers bind to surface sites on the red blood cell to change their density, possibly through cross linking with other polymer complexes or cell components. If this were to occur in platelets too, then some platelets may be functionally removed from binding with fibrin, fibrinogen, and adhesion molecules to alter the platelet component of clot formation, as documented clearly in these two studies. This proposed parallelism between PEG-20k interactions with RBCs and platelets has not been demonstrated empirically but such a nonspecific passivation effect seems reasonable to postulate from the very strong ESR effects of PEG-20k on red cells and from the known affinity of PEG polymers with cell membrane components, including on platelets (19-22). Further studies using fluorescent or electron microscopy imaging may be useful to resolve what is happening to the platelet when PEG-20k is around under clot forming conditions.

In conclusion, this study has expanded our search for a mechanistic explanation for the identified effects of PEG-20k solutions on whole blood coagulation seen in healthy volunteers and trauma patients. We have learned that PEG-20k has little effects on chemical coagulation pathways and on the availability of critical non-catalytic proteins such as fibrinogen and vWF. The effects of PEG-20k solutions on platelet activation may suggest that the predominant effect of these solutions on whole blood clotting at high concentrations may be due to interference with the normal platelet function

during clot activation that mimic a state of mild functional thrombocytopenia, platelet passivation, or thrombasthenia.

FIGURE LEGENDS

Figure 1: Plasma fibrinogen (panel A) and von Willebrand factor (panel B) concentrations in whole blood from healthy volunteers diluted with either saline (1 to 9) or 10% PEG-20k solution (1 to 9). Values are box and whiskers format such that the bar inside of the box is the median value of the sample, the lower and upper borders of the box represent the boundaries between the 1st and 2nd quartile and the 3rd and 4th quartile, respectively, and the ends of the whiskers indicate the population extreme values (low and high). Each group represents 6 individual values. The shaded box is the normal range of values. $P<0.05$, relative to the saline dilutional control group.

Figure 2: The plasma PT (panel A) and aPTT times (panel B) measured in blood from healthy volunteers diluted with either saline (1 to 9) or 10% PEG-20k solution (1 to 9). Values are box and whiskers format such that the bar inside of the box is the median value of the sample, the lower and upper borders of the box represent the boundaries between the 1st and 2nd quartile and the 3rd and 4th quartile, respectively, and the ends of the whiskers indicate the population extreme values (low and high). Each group represents 6 individual values. The shaded box is the normal range of values. $P<0.05$, relative to the corresponding saline dilutional control group. Panel B also shows the effects of micronized silica activator on aPTT compared to activators using kaolin.

Figure 3: Plasma thrombin generation as described by the CAT (Calibrated Automated Thrombogram) test in plasma from healthy volunteers diluted with either saline (1 to 9) or 10% PEG-20k solution (1 to 9). Panel A shows a representative thrombin generation curve showing the major curve attributes used to measure the amount of thrombin generation. Panel B shows the ETF

parameter, which is the area under the thrombin curve from curves generated with volunteer plasma diluted with saline or PEG-20k. Panel C illustrates the peak size of the thrombin curve; Panel D shows the velocity of thrombin generation after activation; Panel E shows the lag time from the time of activation until the start of thrombin generation; and panel F shows the time to peak thrombin activity. Values are box and whiskers format such that the bar inside of the box is the median value of the sample, the lower and upper borders of the box represent the boundaries between the 1st and 2nd quartile and the 3rd and 4th quartile, respectively, and the ends of the whiskers indicate the population extreme values (low and high). Each group represents 6 individual values. The shaded box is the normal range of values. $P<0.05$, relative to the saline dilutional control group.

Figure 4: Platelet mapping studies conducted with whole blood obtained from volunteers diluted with either saline (1 to 9) or 10% PEG-20k solution (1 to 9). The test shows both the inhibition of platelet clot formation (panel A) and the activation of platelet clot formation (panel B) in response to activation with either ADP or arachidonic acid Values are box and whiskers format such that the bar inside of the box is the median value of the sample, the lower and upper borders of the box represent the boundaries between the 1st and 2nd quartile and the 3rd and 4th quartile, respectively, and the ends of the whiskers indicate the population extreme values (low and high). Each group represents 6 individual values. $P<0.05$, relative to the saline dilutional control group.

Figure 5: ADP-induced activation of expression of PAC1 (IIB/IIIA receptor complex) and CD63P (P-selectin) specific receptors on platelets in blood obtained from healthy volunteers diluted with either saline (1 to 9) or 10% PEG-20k solution (1 to 9). Values are box and whiskers format such that the bar inside of the box is the median value of the sample, the lower and upper borders of the box

represent the boundaries between the 1st and 2nd quartile and the 3rd and 4th quartile, respectively, and the ends of the whiskers indicate the population extreme values (low and high). Each group represents 6 individual values.

Figure 6: Erythrocyte Sedimentation Rates (ESR) measured in whole blood obtained from healthy volunteers diluted with saline (1 to 9) containing various concentrations of PEG-20k solution (0%, 7.5%, and 10%) (panel A). The dose dependent effects of PEG-8k on the accelerated ESR effect of 10% PEG-20k are shown in panel B. Values are mean +/- SD, n=4 independent experiments.

Figure 1

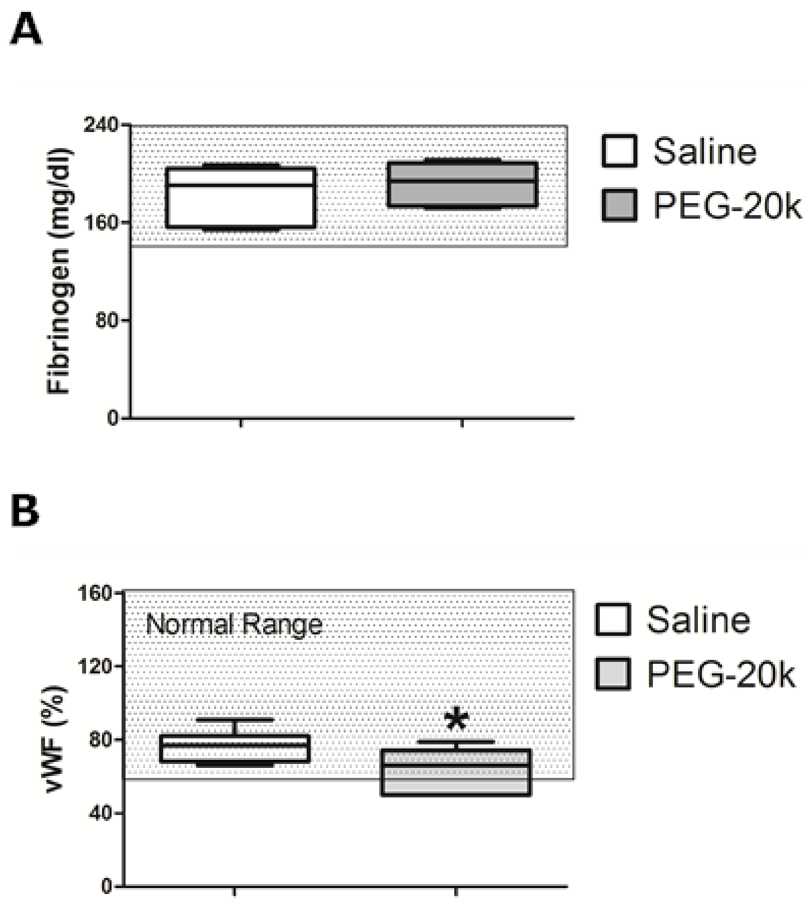

A
240
160
80
0
Fibrinogen (mg/dl)
Saline
PEG-20k
B
160
120
80
40
0
vWF (%)
Normal Range
*
Saline
PEG-20k

Figure 2

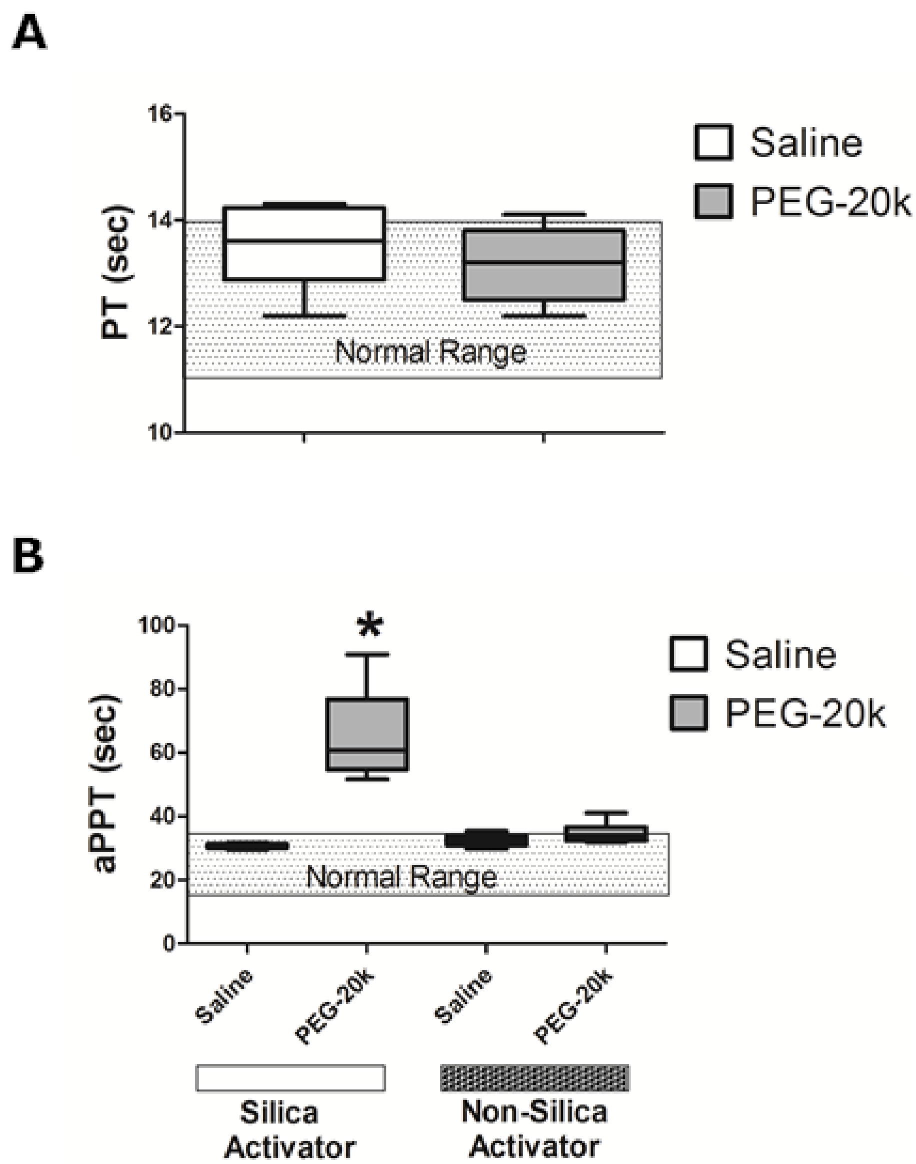

A
16
14
12
10
PT (sec)
Normal Range
Saline
PEG-20k
B
100
80
60
40
20
0
aPPT (sec)
*
Normal Range
Saline
PEG-20k
Saline
PEG-20k
Saline
PEG-20k
Silica Activator
Non-Silica Activator

Figure 3

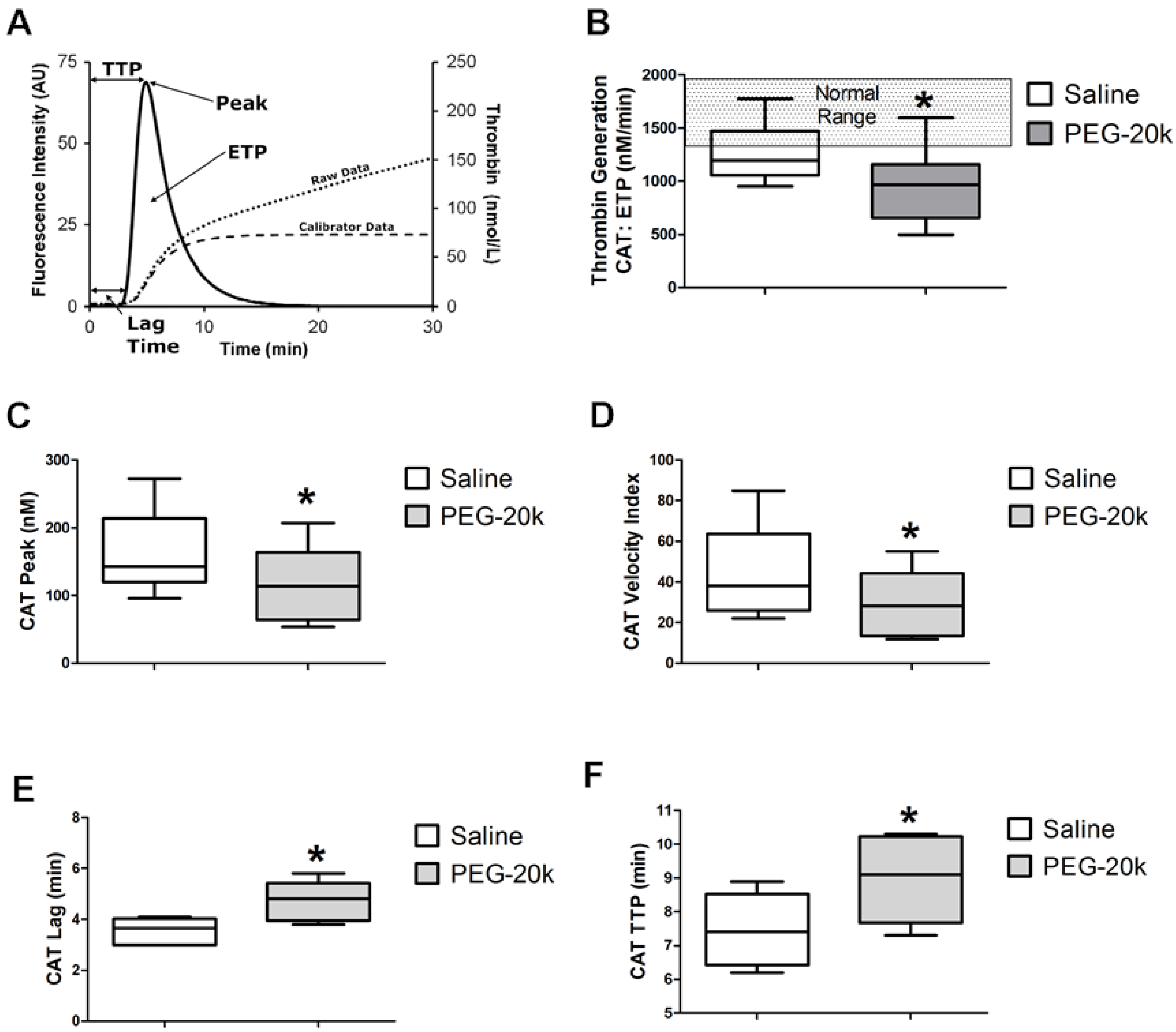
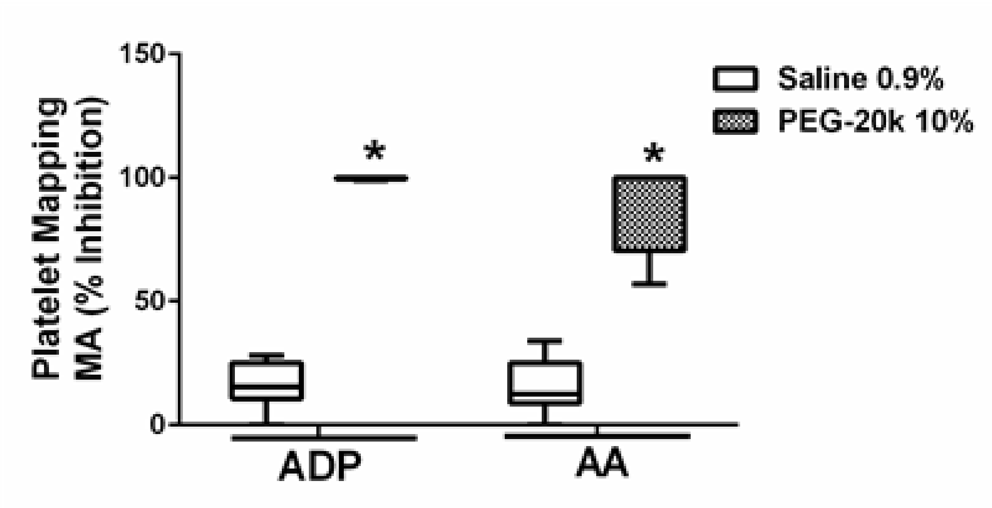

A
TTP
Peak
ETP
Raw Data
Calibrator Data
Fluorescence Intensity (AU)
Thrombin (nmol/L)
Lag Time
Time (min)
B
Thrombin Generation CAT: ETP (nM/min)
Normal Range
*
Saline
PEG-20k
C
CAT Peak (nM)
*
Saline
PEG-20k
D
CAT Velocity Index
*
Saline
PEG-20k
E
CAT Lag (min)
*
Saline
PEG-20k
F
CAT TTP (min)
*
Saline
PEG-20k

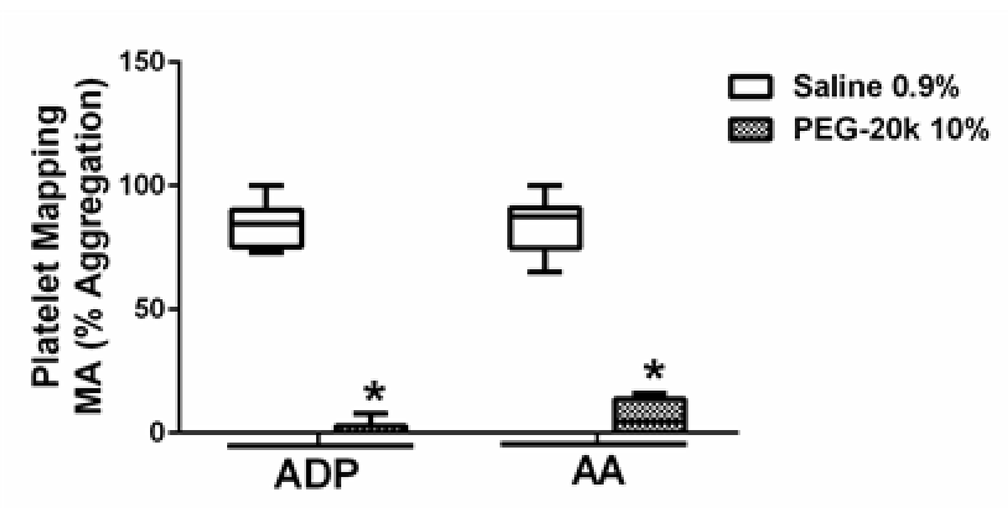
Figure 4

A



Platelet Mapping MA (% Inhibition)
150
100
50
0
Saline 0.9%
PEG-20k 10%
*
*
ADP
AA

B



Platelet Mapping MA (% Aggregation)
150
100
50
0
Saline 0.9%
PEG-20k 10%
*
*
ADP
AA

Figure 5

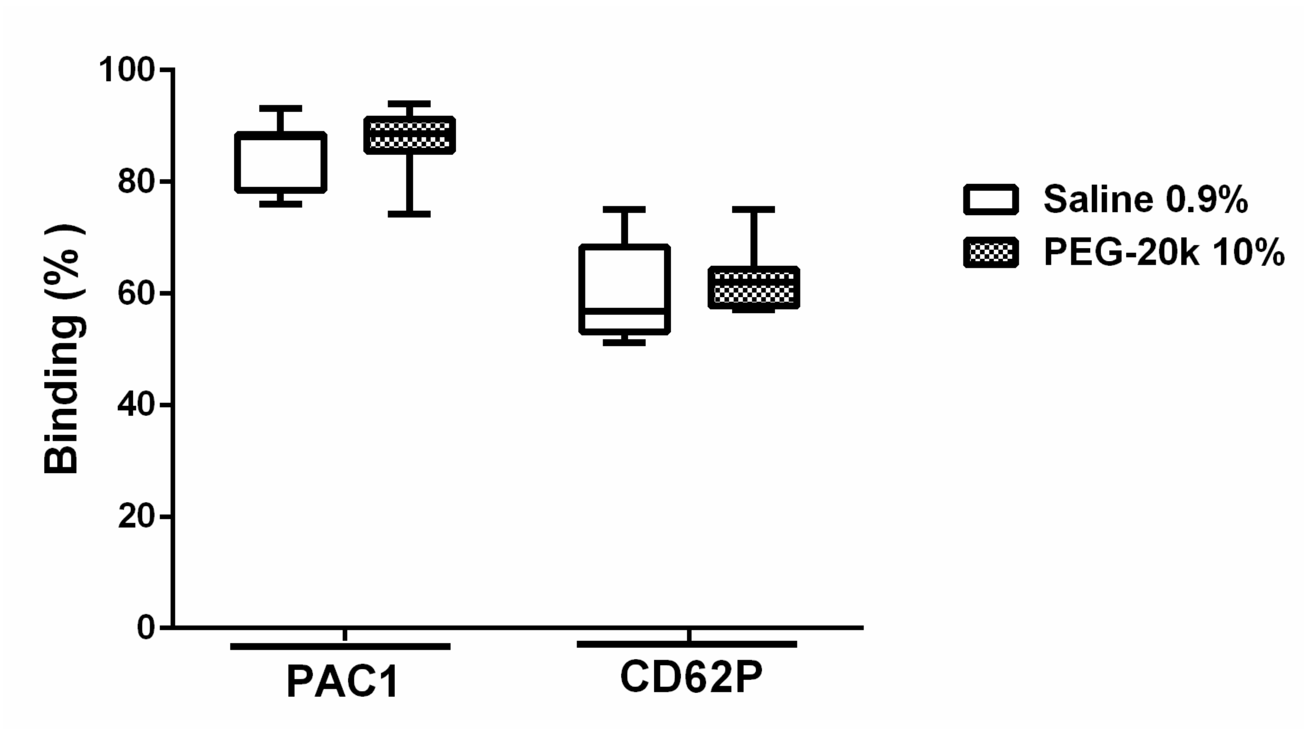
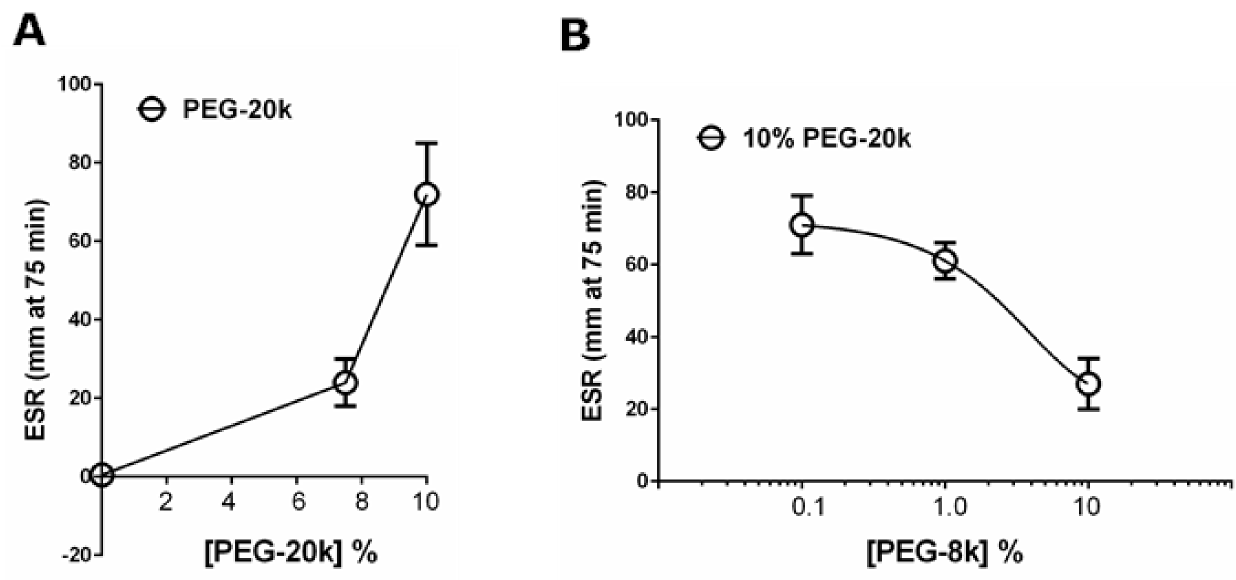


Figure 6



A
PEG-20k
ESR (mm at 75 min)
100
80
60
40
20
0
-20
2
4
6
8
10
[PEG-20k] %
B
10% PEG-20k
ESR (mm at 75 min)
100
80
60
40
20
0
0.1
1.0
10
[PEG-8k] %

References

1. Finkelstein EA, Corso PS, Miller TR. The Incidence and Economic Burden of Injuries in the United States. USA: Oxford University Press; 2006 2006.

2. Kauvar DS, Lefering R, Wade CE. Impact of hemorrhage on trauma outcome: an overview of epidemiology, clinical presentations, and therapeutic considerations. JTrauma. 2006;60(6 Suppl):S3-11.

3. Heckbert SR, Vedder NB, Hoffman W, Winn RK, Hudson LD, Jurkovich GJ, et al. Outcome after hemorrhagic shock in trauma patients. JTrauma. 1998;45(3):545-9.

4. Franklin GA, Boaz PW, Spain DA, Lukan JK, Carrillo EH, Richardson JD. Prehospital hypotension as a valid indicator of trauma team activation. JTrauma. 2000;48(6):1034-7.

5. van Lambalgen AA, van den Bos GC, Thijs LG. Whole body plasma extravasation in saline and Haemaccel loaded rats: effects of endotoxemia. IntJMicrocircClinExp. 1990;9(3):303-18.

6. Parrish D, Plant V, Lindell SL, Limkemann A, Reichstetter H, Aboutanos M, et al. New low-volume resuscitation solutions containing PEG-20k. JTrauma Acute Care Surg. 2015;79(1):22-9.

7. Stein DM, Scalea TM. Capillary leak syndrome in trauma: what is it and what are the consequences? AdvSurg. 2012;46:237-53.

8. Duchesne JC, McSwain NE, Jr., Cotton BA, Hunt JP, Dellavolpe J, Lafaro K, et al. Damage control resuscitation: the new face of damage control. JTrauma. 2010;69(4):976-90.

9. Riha GM, Kunio NR, Van PY, Hamilton GJ, Anderson R, Differding JA, et al. Hextend and 7.5% hypertonic saline with Dextran are equivalent to Lactated Ringer's in a swine model of initial resuscitation of uncontrolled hemorrhagic shock. JTrauma. 2011;71(6):1755-60.

10. Riha GM, Kunio NR, Van PY, Kremenevskiy I, Anderson R, Hamilton GJ, et al. Uncontrolled hemorrhagic shock results in a hypercoagulable state modulated by initial fluid resuscitation regimens. JTrauma AcuteCare Surg. 2013;75(1):129-34.

11. Cotton BA, Guy JS, Morris JA, Jr., Abumrad NN. The cellular, metabolic, and systemic consequences of aggressive fluid resuscitation strategies. Shock. 2006;26(2):115-21.

12. Plant V, Limkemann A, Liebrecht L, Blocher C, Ferrada P, Aboutanos M, et al. Low-volume resuscitation using polyethylene glycol-20k in a preclinical porcine model of hemorrhagic shock. The journal of trauma and acute care surgery. 2016;81(6):1056-62.

13. Parrish D, Lindell SL, Reichstetter H, Aboutanos M, Mangino MJ. Cell Impermeant-based Low-volume Resuscitation in Hemorrhagic Shock: A Biological Basis for Injury Involving Cell Swelling. Ann Surg. 2016;263(3):565-72.

14. Plant V, Parrish D, Limkemann A, Ferrada P, Aboutanos M, Mangino MJ. Low Volume Resuscitation for Hemorrhagic Shock: Understanding the Mechanism of PEG-20k. J Pharmacol Exp Ther. 2017.

15. Mangino MJ, Liebrecht LK, Plant V, Limkemann A. Crystalloid and Colloid Resuscitation: Hypertonic Saline, Starches, Polymers, and Gelatins. In: Pascual JL, Cannon JW, editors. Hemorrhagic Shock: Recognition, Pathophysiology, and Management. Hauppauge, NY: Nova Publishers; 2017.

16. Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E, Wagenvoord R, et al. Calibrated automated thrombin generation measurement in clotting plasma. Pathophysiol Haemost Thromb. 2003;33(1):4-15.

17. Bochsen L, Wiinberg B, Kjelgaard-Hansen M, Steinbruchel DA, Johansson PI. Evaluation of the TEG platelet mapping assay in blood donors. Thromb J. 2007;5:3.

18. Schmitz G, Rothe G, Ruf A, Barlage S, Tschope D, Clemetson KJ, et al. European Working Group on Clinical Cell Analysis: Consensus protocol for the flow cytometric characterisation of platelet function. Thromb Haemost. 1998;79(5):885-96.

19. McNamee CE, Yamamoto S, Higashitani K. Effect of the physicochemical properties of poly(ethylene glycol) brushes on their binding to cells. Biophys J. 2007;93(1):324-34.

20. Wee YM, Lim DG, Kim YH, Kim JH, Kim SC, Yu E, et al. Cell surface modification by activated polyethylene glycol prevents allosensitization after islet transplantation. Cell Transplant. 2008;17(10-11):1257-69.

21. Israelachvili J. The different faces of poly(ethylene glycol). Proc Natl Acad Sci U S A. 1997;94(16):8378-9.

22. Sauter A, Richter G, Micoulet A, Martinez A, Spatz JP, Appel S. Effective polyethylene glycol passivation for the inhibition of surface interactions of peripheral blood mononuclear cells and platelets. Biointerphases. 2013;8(1):14.